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Breast Cancer

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13. ABSTRACT (Maximum 200 Words) We have developed a safety modified adenoviral vector in which the tumor specific L-plastin promoter can be used to control the expression of therapeutic genes such as cytosine deaminase (CD). The CD gene product catalyzes the conversion of a non-toxic precursor, 5-Fluorocytosine (5FC) into a drug, 5-Fluorouracil (5FU), thereby sensitizing the cells in which it is produced to 5FC induced toxicity. We have also generated vectors in which the L-plastin promoter is used to drive the expression of the viral E1A gene, which the adenovirus needs to replicate within mammalian cells. The replication of a virus within a mammalian cell takes over all of the metabolic and cell proliferative machinery of the cells and thereby kills it. Our data shows that the adenoviral vector which carries the L-plastin-E1A transcription unit can replicate within breast cancer cells and other estrogen dependent carcinomas, such as ovarian cancer, while not being able to replicate in normal cells. This L-plastin-E1A vector can suppress the growth of human breast cancer cell lines in immunosuppressed mice. We have recently isolated a vector in which both the CD E1A genes are under the control of the L-plastin promoter, which is currently under study.				
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Introduction:

This report summarizes progress on a project funded by the US Army Breast Cancer Research Fund (DAMD17-00-9457) which is designed to develop a new approach for the treatment of metastatic breast cancer. We have engineered a cold virus (adenovirus) so that it becomes specifically toxic to tumor cells but is not toxic to normal cells. This is based on the introduction a tumor specific transcriptional switch (promoter) in front of (5') to the E1A gene of the virus. The E1A gene controls the ability of the virus to replicate itself within the cells of the body. This vector is predicted not to replicate within normal cells but to replicate within the tumor cells and thereby to kill them. We have developed such a safety modified virus in which the tumor specific L-plastin promoter can be used to control the expression of therapeutic genes such as cytosine deaminase (CD). The CD gene product catalyzes the conversion of a non-toxic precursor, 5-Fluorocytosine (5FC) into a toxic drug, 5-Fluorouracil (5FU), thereby sensitizing the cells in which it is produced to 5FC induced toxicity. We have developed an adenoviral vector in which the L-plastin promoter is used to drive the expression of the viral E1A gene, which the adenovirus needs to replicate within mammalian cells. The replication of a virus within a mammalian cell takes over all of the metabolic and cell proliferative machinery of the cells and thereby kills it. The data presented within this proposal shows that this vector can replicate within breast cancer cells and other estrogen dependent carcinomas, such as ovarian cancer, while not being able to replicate in normal cells. This vector is toxic to breast cancer cells and not toxic to normal cells. We have shown that this vector can suppress the growth of human breast cancer cell lines in immunosuppressed mice. We have recently developed another adenoviral vector in which both the E1A and the CD genes are under control of the L-plastin promoter. This vector is currently under study.

Body: Narrative Description of the Results During the Reporting Period of Funding: July 1, 2000-June 30, 2001:

The goal of our project is to design, build and test the efficacy of adenoviral vectors which are directly toxic for breast cancer cells on the basis of their ability to multiply within these cells, and thereby cause their lysis.

In order to make this vector totally specific for the breast cancer cell and therefore to spare the normal cells of the body, we placed a regulatory switch within the virus that causes it to multiply within the breast cancer cell leading to the death of these cells. This switch is called the L-plastin promoter. In the initial period of funding by the US Army BCRF, Injae Chung and her coworkers of the Deisseroth laboratory, in collaboration (see Appendix #1), showed that this L-plastin molecular switch could turn on genes in breast and ovarian cancer cell lines, but did not activate genes within the non cancerous cells of the body. This work has been published in *Cancer Gene Therapy*, 1999. During the US Army funding period, Xue yuan Peng of the Deisseroth laboratory, also showed that the L-plastin promoter could selectively activate genes in adenoviral vectors in cancer cells present in animals as well in cancer cells in tissue culture. This paper has been published in *Cancer Research* (2001), and is presented as Appendix #2. An adenoviral vector carrying one of these genes, the cytosine deaminase gene, under the control of the L-plastin promoter, was shown in vivo and in vitro to sensitize the breast cancer and ovarian cancer cells to 5-Fluorocytosine (5FC). This vector did not support the expression of genes carried by the adenoviral vector in normal tissues corresponding to the cancer tissue in which the vector was active. Thus, these vectors were selective for cancer cells. In the manuscript from our laboratory by X.Y. Peng et al, describing this work was published in *Cancer Research* (see Appendix #2), the US Army support was recognized.

On the basis of this of this information, Dr. Lixin Zhang of our laboratory decided to use the L-plastin molecular switch to regulate the E1A gene, which the virus needs to replicate itself in mammalian cells. He successfully constructed such a vector (Ad-Lp-E1A). As outlined above, continued replication of an adenovirus within a cell can lead to its death. Since this switch is active in cancer cells but not in normal cells, the prediction is that the virus so generated (called Ad-Lp-E1A) would be toxic for breast cancer cells but not for normal non cancerous tissue and cell lines.

For comparison, Dr. Zhang introduced an activating switch called the CMV promoter in front of the E1A gene of the adenovirus that was active in both normal and cancerous tissue. This virus is called the Ad-CMV-E1A vector. In addition, he created a virus which lacked the E1A gene, and therefore could not replicate, but contained the CMV molecular switch driving a gene which does not produce replication of the virus, thereby making the virus non-toxic for any cell. This inactive negative control virus, which is predicted to be non toxic to either cancer or normal cells, is called Ad-CMV-LacZ. Finally, Dr. Zhang placed a molecular switch called the Tyrosinase promoter in front of the E1A viral replication gene (this virus is called Ad-Tyr-E1A). The prediction is that this virus will be multiply not in breast cancer cells but would replicate only in a specific type of skin cell,

called the melanin producing cell or the melanocyte in which the tyrosinase switch is normally active. The resulting vector would replicate within the melanoma cell and kill it. Dr. Zhang compared all of these synthetic viruses with the naturally occurring adenovirus, which can destroy human cells by multiplying, overwhelming and destroying the normal cells of the body. This virus is called Ad5. The organization of these viral vectors is outlined below in Figure 1.

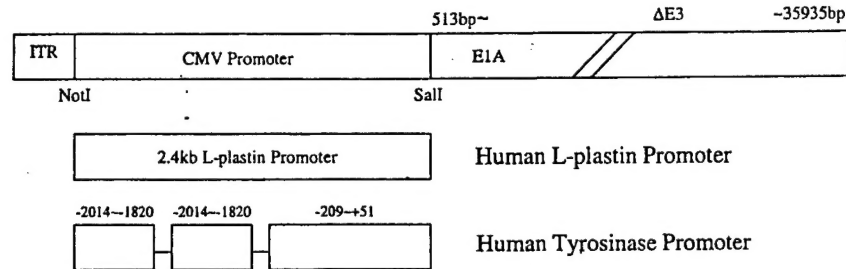


Figure 1: Structure of the E1A switches in the viruses.

Dr. Zhang then tested each one of these vectors to determine which of them drove the expression of the E1A high enough to push the vector to kill cancer cells. The vectors tested included the Ad-Lp-E1A vector, which contained the breast cancer specific L-plastin switch, which should be producing E1A in the breast cancer cells (MDA-MB-468), but not within the fibroblast cells (CCD), the Ad-Tyr-E1A vector, which should be active in melanoma cells, but not in breast cancer cells nor in the CCD fibroblast cell line, and the naturally occurring adenovirus, Ad5, which should be actively producing E1A in both the breast cancer and the CCD cell lines. As shown in Figure 2, the naturally occurring adenovirus, Ad5 actively produced E1A in both the breast cancer cell line (see lane 5 of Figure 2) and the CCD fibroblast cell line (see lane 4 of Figure 2) as expected. The tyrosinase driven vector (Ad-Tyr-E1A) did not produce detectable E1A protein in either the breast cancer cell line (see lane 1 of Figure 2) nor in the CCD fibroblast cell line (see lane 2 of Figure 2) as predicted. Finally, the vector in which E1A expression was controlled by the L-plastin breast cancer specific promoter or switch, generated very high levels of the E1A protein within the MDA-MB-468 breast cancer cell line (see lane 3 of Figure 2) and did not produce detectable levels of the E1A protein within the CCD fibroblast cell line (see lane 6 of Figure 2). Thus, the vectors were behaving just as designed. The results of these experiments suggested that the Ad-Lp-E1A virus would be toxic to breast cancer cells, but to non cancerous cells.



Figure 2. Western blot of E1A Expression. Ad5 E1A protein expression in MDA-MB-468 cells and CCD fibroblast cells following infection with Ad-Tyr-E1A (lane 1, 2), Ad-Lp-E1A (lane 3,6) or wildtype adenovirus 5 (lane 4,5) was determined by Western blot analysis. Lane 1, 3, 5 are MDA-MB-468 cells. Lane 2, 4, 6 are CCD cells.

Dr.Zhang then added the Ad-Lp-E1A vector to cultures of the breast cancer cell line, MDA-MB-468. As shown in Figure 3 below, the vector caused the breast cancer cells to round up and lift off of the culture dish (see the cells in the middle and left hand panels of Figure 3) which suggests that the cells are dying. The MDA-MB-468 cells not exposed to the vector did not show such changes in culture (see the right hand panel in Figure 3).

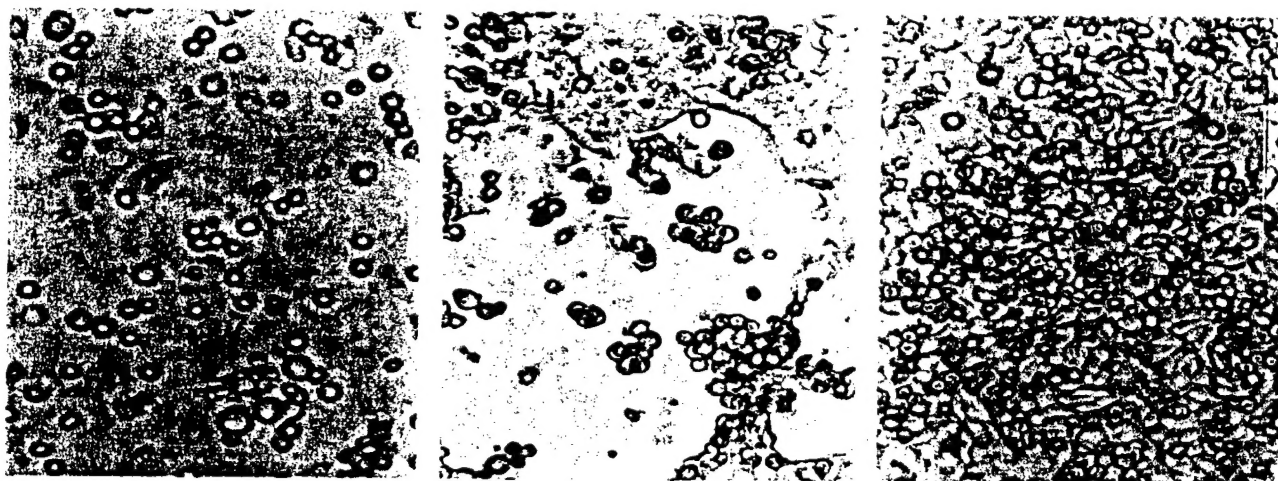
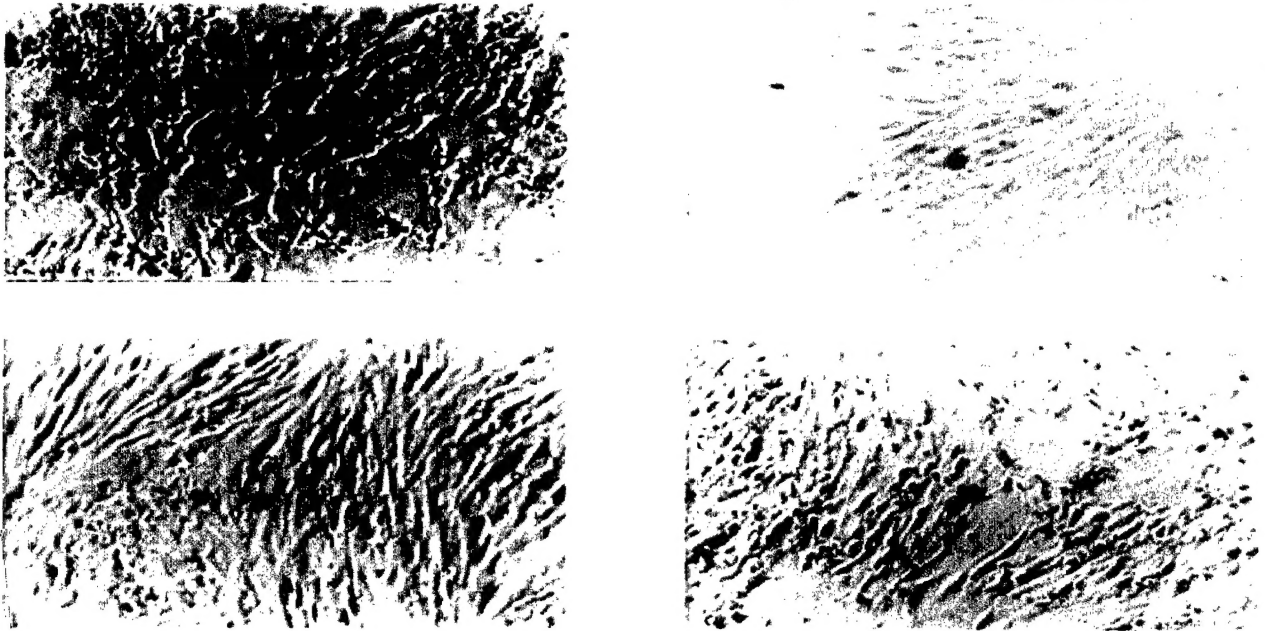


Figure 3: Effect of the Ad-Lp-E1A vector on the MDA-MB-468 Breast Cancer Cell Line.

Dr. Zhang then studied the effect of each of these vectors on the CCD non-cancerous cell line. Since the Ad-Lp-E1A and Ad-Tyr-E1A vectors did not produce the E1A protein in the CCD cell line (see Figure 2), the prediction was that these vectors would not be toxic to the CCD cell line. As shown in Figure 4 below, there are no visible signs of toxicity in the CCD cell line following exposure to the Ad-Lp-E1A (right upper panel) or to the Ad-Tyr-E1A (left lower panel) vectors, while both the naturally occurring virus, Ad5 (left upper panel), and the tumor non specific virus, Ad-CMV-E1A (right lower panel), cause the CCD cells to lift off and to round up. Therefore, this data shows that the Ad-Lp-E1A virus is specifically toxic to breast cancer cell lines but not to non-cancerous cell lines. Figure 4: Effect of Vectors on the CCD Non-Cancerous Cell line.



Dr. Zhang then tested the effect of the Ad-Lp-E1A on fresh ovarian cancer cells taken from a patient. As shown in Figure 5, the Ad-Lp-E1A virus causes the cancer cells to round up and die (middle and righthand panels), whereas the cells not exposed to the vector do not round up in culture (lefthand panel).

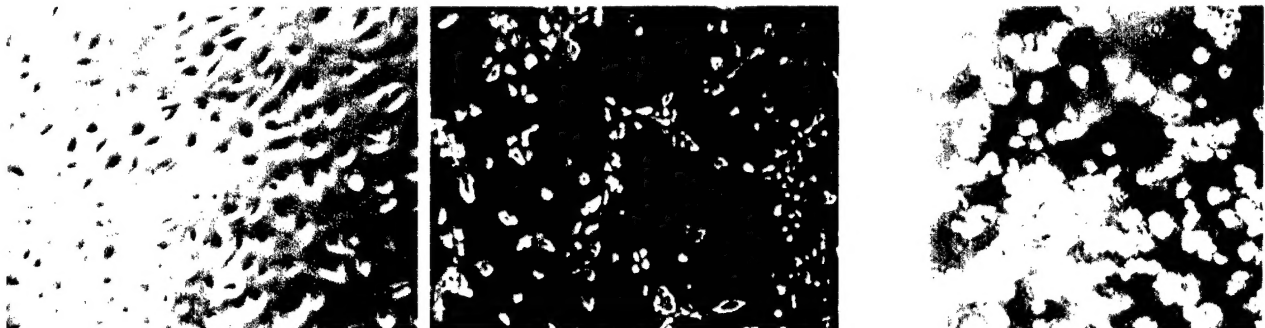
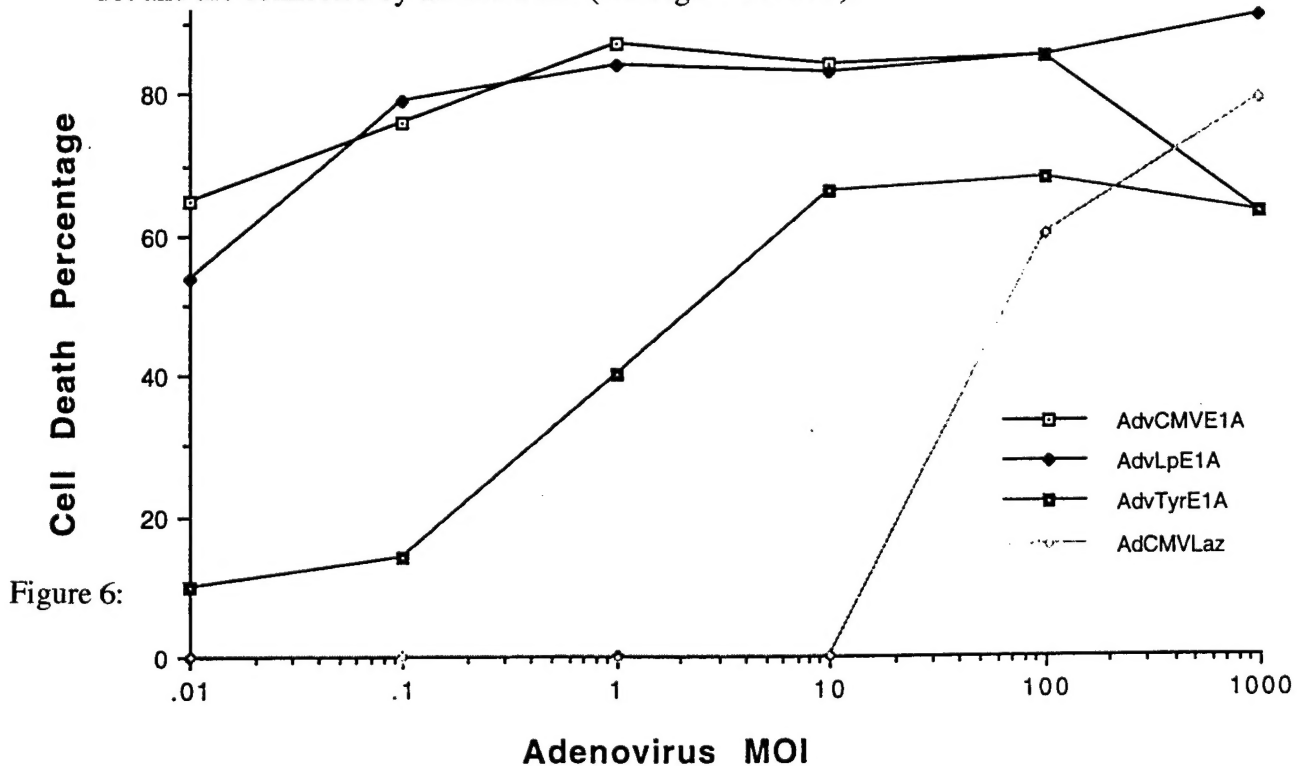


Figure 5: Effect of the Ad-Lp-E1A virus on Fresh Estrogen Dependent Cancer Cells.

Then, Dr. Zhang compared the toxic effect of the four vectors: the breast cancer specific vector (Ad-Lp-E1A), the melanoma specific vector (Ad-Tyr-E1A), the positive control vector which should be non-selectively toxic to both normal and cancerous cells (Ad-CMV-E1A), and the negative control vector which should not be toxic to any cells (Ad-CMV-LacZ). As shown in Figure 6 below, the Ad-Lp-E1A vector (solid diamonds connected by the solid line), which is designed to be selectively toxic to breast cancer cells but to spare normal cells, is 1000 fold more toxic to the primary ovarian cancer cells than is the negative control vector (Ad-CMV-LacZ), which is designated by the open diamonds connected by the dotted line. In spite of the specificity of the Ad-Lp-E1A vector for breast cancer cells, it is as actively toxic as the tumor non specific positive control virus, Ad-CMV-E1A, which is designated by the open squares which contain a dot and are connected by the solid line (see Figure 6 below).



As a final test of specificity, Dr. Zhang compared the toxicity of the Ad-Lp-E1A vector with that of the Ad-Tyr-E1A vector to melanoma cells. As shown below in Figure 7, the Ad-Lp-E1A vector (right upper panel) was not toxic to the human TF-2 melanoma cell line whereas the Ad-Tyr-E1A vector (left lower panel) was toxic to these cells, as expected. The effect of the unmodified adenovirus (right lower panel) and the Ad-CMV-E1A vector (left upper panel) is shown to be toxic to the human TF-2 cells.



Figure 7: Effect of the Vectors on the TF-2 Melanoma Tumor Cell Line.

Dr. Zhang then tested the efficacy of the breast cancer specific vector (Ad-Lp-E1A) and the melanoma specific vector (Ad-Tyr-E1A) in human breast cancer cells (MDA-MB-468) growing as subcutaneous nodules in immunodeficient (SCID) mice. As shown in Figure 8, the injection of the Ad-Lp-E1A vector into a subcutaneous tumor mass made up of the MDA-MB-468 tumor cells stopped the growth of these cells and made them regress in the SCID mouse. Similarly, the Ad-Tyr-E1A vector suppressed the growth of the human TF-2 melanoma cell line in the SCID mouse (see Figure 9 below).



Figure 8A: Photograph of Effect of Injection of the Ad-Lp-E1A or the Negative Control Virus, Ad-CMV-LacZ, into tumor nodules of the human breast cancer cell line MDA-MB-468 in the SCID mouse.

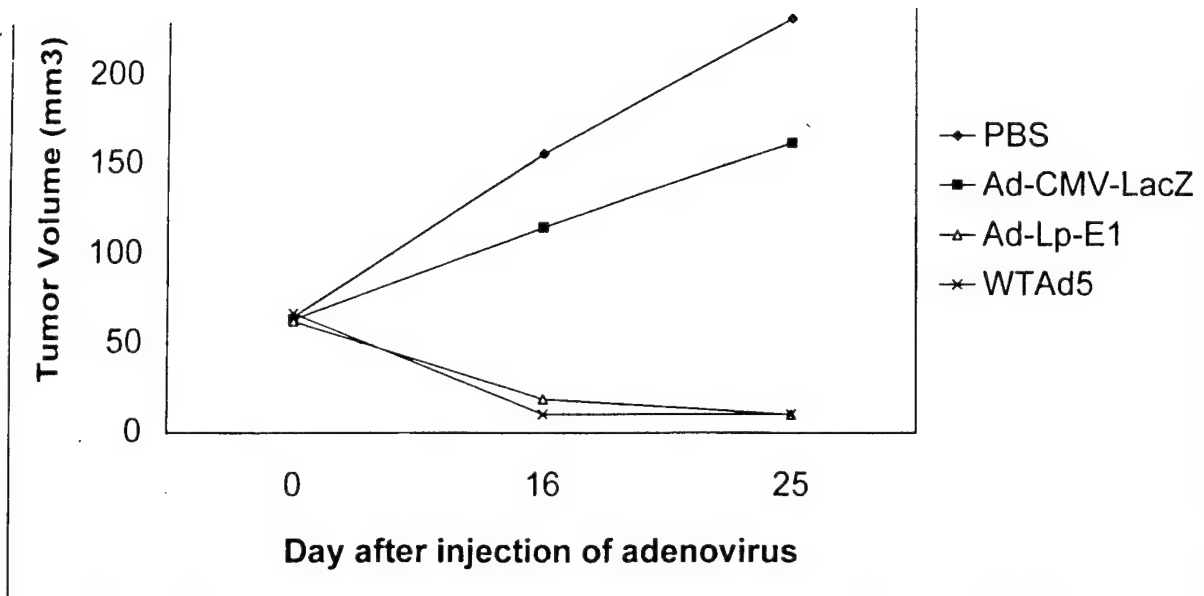


Figure 8B: Regression of the Human MDA-MB-468 Breast Cancer Cell Line in the SCID Mouse Following Injection with the Ad-Lp-E1A and Other Vectors.

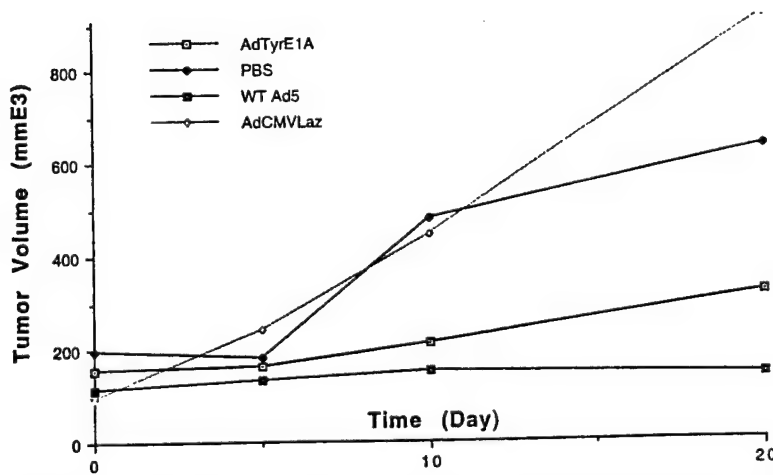


Figure 9: Effect of Injection of the Ad-Tyr-E1A Melanoma Cell Line into the TF-2 Human Melanoma Cell Line in the SCID Mouse.

Key Research Accomplishments:

- a. Development of a of replication competent adenoviral vector which contains a tumor specific promoter (L plastin) controlling the expression of the E1A viral gene which is necessary for viral replication in breast cancer cells.
- b. Demonstration that these vectors are selectively and directly toxic to breast and ovarian cancer cell lines in vitro.
- c. Demonstration in an animal model that these vectors can suppress the growth of human breast cancer cell lines in immunocompromised mice
- d. Design, construction and isolation of a new series of vectors which contain in addition to the E1A gene, the cytosine deaminase gene under control of a tumor specific promoter (the L plastin promoter).

Reportable Outcomes:

a. Manuscripts:

1. Peng XY, Rutherford, T, Won JH, Pizzorno G, Sapi E, Kacinski, B, Leavitt, J, Fujii T, Crystal R, and Deisseroth, A. The use of the L-plastin promoter for adenoviral mediated tumor-specific gene expression in ovarian and bladder cancer cell lines. *Cancer Research* 61:4405-4413, 2001 . This paper is attached as Appendix #2.
2. Zhang, L, Peng, XY, Pizzorno, G, and Deisseroth, A. Tumor specific promoters confer tumor specific cytolytic action on adenoviral vectors in estrogen dependent tumors and melanoma. Submitted to *Molecular Therapy*, 2001. This paper is attached as Appendix #3.
3. Deisseroth, A. et al. Molecular Chemotherapy, in supplement on Cytotoxic Drug Therapy. Ed. Peter Boszc. *Journal of Gynecologic Oncology*, in Press, 2001. This paper is attached as Appendix #4.

b. Development of Vectors:

- a. Vectors which contain the E1A gene governed by the breast cancer specific L plastin promoter and are selectively toxic to human breast cancer cell lines growing as subcutaneous deposits in SCID mice.
- b. Vectors which contain double transcription units which contain the cytosine deaminase chemotherapy sensitization gene along with the E1A viral replication gene under control of the breast cancer specific transcriptional promoter, L plastin.

Conclusions:

This data shows that the Ad-Lp-E1A is selectively toxic to human breast cancer cells growing in culture or in animals. This suggests that this vector might be useful in the treatment of breast cancer resistant to chemotherapy or to hormonal therapy. On the basis of this work, a manuscript summarizing these findings was recently submitted to the journal, *Molecular Therapy*, which is the official journal of the American Society of Human Gene Therapy. A copy of this manuscript, which acknowledges the support of the US Army, is enclosed with the progress report (see Appendix #3).

References: None

Appendices

- 1. Published paper by Chung et al, Published in Clinical Cancer Research in 1999.**
- 2. Manuscript by Peng et al Published in Cancer Research, 61: 4405-4413, 2001.**
- 3. Manuscript submitted to Molecular Therapy by Zhang et al, 2001.**
- 4. Manuscript in press in Journal of Gynecologic Oncology.**

Use of L-plastin promoter to develop an adenoviral system that confers transgene expression in ovarian cancer cells but not in normal mesothelial cells

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The objective of this study was to develop an adenoviral vector system that would generate a pattern of expression of exogenous therapeutic genes appropriate for the treatment of ovarian cancer. For this purpose, we have generated a replication-deficient recombinant adenoviral vector, AdLPLacZ, which contains the human L-plastin (LP) promoter (LP-P) driving the *Escherichia coli* LacZ gene. LP is constitutively expressed at high levels in malignant epithelial cells but is not expressed in normal tissues, except at low levels in mature hematopoietic cells. Because adenoviral vectors infect early hematopoietic multilineage precursor cells only poorly or not at all, this vector would be of use in the peritoneal cavity and *in vitro* for marrow purging. We first analyzed the expression of the LacZ reporter gene in ovarian and breast cancer cell lines, normal fibroblasts, and leukemia cell lines using the adenoviral vector in which the LacZ gene is governed by the LP-P promoter (AdLPLacZ) or in which the LacZ gene is governed by the cytomegalovirus (CMV) promoter (AdCMVLacZ). We found equivalent and high levels of expression of β -galactosidase (β -gal) by AdLPLacZ and AdCMVLacZ vectors in the breast or ovarian cancer cell lines as well as in a fibrosarcoma cell line, indicating that the adenoviral vectors infected these cells and expressed their transgenes equally with the LP and CMV promoters. Expression of the LacZ gene with the CMV vector but not with the LP-P vector was observed in experiments with normal fibroblasts, indicating that the vectors infected the cells, but that the LP-P was not active within them. In hematopoietic cells such as U937 cells, no measurable β -gal activity was detected in cells infected either by AdLPLacZ or by AdCMVLacZ, indicating that the adenoviral vectors were not infecting the cells. Although β -gal activity was observed in fresh ascitic ovarian cancer cells after infection with adenoviral vectors containing CMV or the LP promoters, β -gal activity was detected in a portion of a biopsy of normal peritoneum when the tissues were exposed to the AdCMVLacZ vector, but not when tissues were exposed to the AdLPLacZ vector. These results suggest that the transcription of therapeutic genes in cells infected by the AdLP vectors would be restricted to LP expression-positive ovarian carcinoma cells but would not be seen in the normal mesothelial cells of the peritoneal cavity. This possibility implies that adenoviral vectors carrying therapeutic genes driven by the LP-P would be of use for the intracavitary treatment ovarian cancer.

Key words: Ovarian cancer; LacZ; L-plastin; tumor specific.

Gene therapy represents a potentially novel approach to cancer treatment, in which the transfer of genetic material into a specific cell type alters the phenotype of the target cells¹⁻³ in a way that improves the outcome of therapy. Adenoviral vectors have become the most widely used vector for the delivery of prodrug activation transcription units in the field of

cancer gene therapy.⁴⁻⁸ However, one of the limitations of this vector system for cancer gene therapy may be its broad cellular host range, which results in toxicity to both the tumor cells and the surrounding normal cells. One way to circumvent this limitation would be through the use of a tumor- or tissue-specific promoter for the therapeutic gene carried by the vector, which is active in the target tumor cells but not in the normal cells. Comparative examination studies of protein synthesis in normal and neoplastic human fibroblasts that were conducted in the laboratory of J.L.⁹⁻¹² led to the discovery of *L-plastin* (LP), a gene that codes for an actin-binding protein, which is expressed at high levels in human epithelial cancer cells.

In their review of solid tumors, LP was found to be a marker expressed at high levels in the majority of human

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cancer cells of nonhematopoietic origin.⁹⁻¹² A survey of simian virus 40-transformed human fibroblasts and human sarcoma cell lines as well as human carcinoma cell lines has demonstrated that the *LP* gene was transcriptionally active, although at widely varying degrees, in nearly all of the human cancer cells tested.⁹⁻¹² In particular, high levels of *LP* synthesis are found in tumors derived from female reproductive organs.¹² In addition, the *LP* gene seems to be transcriptionally regulated through the 5.1 kilobases (kb) of the 5'-regulatory region.¹¹⁻¹² Because the adenoviral vector has a wide host range in epithelial cells, we proposed using the *LP* promoter (LP-P) in an adenoviral vector to restrict the expression of the adenoviral vector therapeutic gene to the *LP*-positive carcinoma cells, so as to avoid expression in normal cells. In the present study, we generated a replication-deficient adenoviral vector carrying the *LacZ* gene in a transcriptional unit governed by a truncated LP-P (a 2.4-kb subset of the 5'-promoter region of the *LP* gene) to accommodate both the LP-P and therapeutic genes. We subsequently studied the use of this LP LacZ adenoviral vector in various human cell lines and patient samples to determine the pattern of its transgene expression in neoplastic and normal cells.

MATERIALS AND METHODS

Generation of AdLPLacZ

We obtained a replication-incompetent adenoviral type 5-based vector from which the E1 and E3 genes were deleted (provided by the laboratory of R.G.C., The New York Hospital, Cornell Medical Center).³⁻⁵ The AdLPLacZ vector is a modification of the vector obtained from this laboratory, in which the human LP-P and the *Escherichia coli* *LacZ* gene that codes for β -galactosidase (β -gal) have been introduced by the Deisseroth laboratory into the E1 region. The first step in the generation of AdLPLacZ was to construct an E1 shuttle vector, pLPLacZ, which contains the expression cassette of the *LacZ* gene under the control of the human LP-P. This was accomplished as follows: we first isolated a *ScaI* 2.4-kb fragment (i.e., -2265 to +118 base pairs (bp)) of the human LP-P from the pHLPr- β -gal-Neo plasmid, which was obtained from J.L. (Palo Alto Medical Research Institute),⁹⁻¹² as shown in Figure 1. At the beginning of the construction, the vector was identical with that outlined by Hirschowitz et al.⁵ We then ligated the *ScaI* 2.4-kb fragment to the *HincII* site of pBluescript II SK⁻ (Stratagene, La Jolla, Calif), to produce pLPSK (see Fig 1). The *SpeI/XhoI*-digested 2.4-kb fragment of pLPSK was then ligated to the *SpeI/XhoI*-digested pCMV β -P (obtained from R.G.C.)³⁻⁵ after removing the cytomegalovirus (CMV) enhancer/promoter to obtain pLPLacZ (see Fig 1). The adenovirus vector AdLPLacZ was produced by homologous recombination using standard techniques.³ For homologous recombination to occur, pBHG10 (Microbix Biosystems, Ontario, Canada) and pLPLacZ were cotransfected into 293 cells by the calcium phosphate coprecipitation method. Individual plaques were screened, and the identity of each as AdLPLacZ was verified (see Fig 2) by amplifying part of the sequences of the *LacZ* gene and Ad5 by polymerase chain reaction (PCR). The sequences of the primers used for the screen were as follows: the forward primer of Ad5, 5'-TCGTTTCTCAG-CAGCTGTTG-3', and the reverse primer of Ad5, 5'-CATCT-

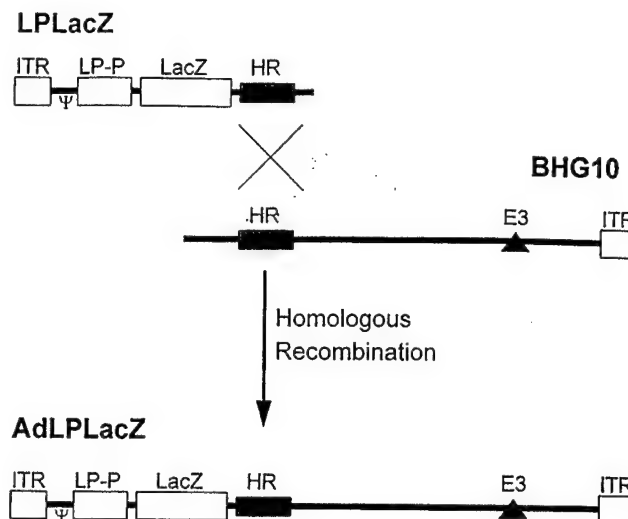


Figure 1. Assembly of AdLPLacZ adenoviral vector by the homologous recombination method. LPLacZ is a shuttle vector that contains an adenoviral inverted terminal repeat (ITR) and packaging signal (Ψ), the *LacZ* gene driven by the LP-P, and some adenoviral vector sequences that are homologous in sequence (HR) to those present in the BHG10 adenoviral vector plasmid clone. These HR sequences, when combined with the pBHG10 adenoviral vector plasmid, produce a complete replication-incompetent adenoviral vector (AdLPLacZ), which contains the *LacZ* gene driven by the LP-P. The BHG10 plasmid was obtained from the laboratory of Frank Graham. (Microbix Biosystems, Ontario, Canada) It contains all of the adenoviral genes 3' to the E1 gene, except for the E3 gene, which has been deleted. There is another ITR at the very 3' end of the vector. The complete recombinant vector is designated AdLPLacZ.

GAAGTCAAAGCGTGG-3', were located at 11 and 13.4 map units of the Ad5 genome, respectively, which generated a 860-bp PCR product as published previously.¹³ The primer set for the *LacZ* gene was designed according to the sequence information for the pCMV β -gal plasmid provided by Clontech (Palo Alto, Calif). The forward primer of *LacZ* gene included from -2072 to -2092 bp (5'-CCTGCTGATGAAGCA-GAAC-3'); the reverse primer was from -2340 to -2358 bp (5'-CGATTAGTGCTGCGGACA-3'), which generated a 287-bp PCR product. The results of the PCR assays (see Fig 2) documented the introduction of the *LacZ* transcription unit into the adenoviral vector.

AdLPLacZ and control virus (AdCMVLacZ), which contained a CMV promoter driving the *LacZ* gene (obtained from the laboratory of R.G.C.), were propagated in 293 cells and recovered 36 hours after infection by five cycles of freezing/thawing of the infected cells. All viral preparations were purified by CsCl density centrifugation, dialyzed, and stored in dialysis buffer (10 mM tris(hydroxymethyl)aminomethane-HCl (pH 7.4) and 1 mM MgCl₂) at -70°C before use. Titers of the viral stocks were determined by plaque assay using 293 cells by standard methods.¹⁴

Cell culture

Cells were maintained in Iscove's modified essential medium (Biofluids, Rockville, Md) supplemented with 10% fetal bovine sera (FBS) and 2 mM glutamine (in the case of 293 cell line); in Dulbecco's modified Eagles' medium/F12 (Life Tech-

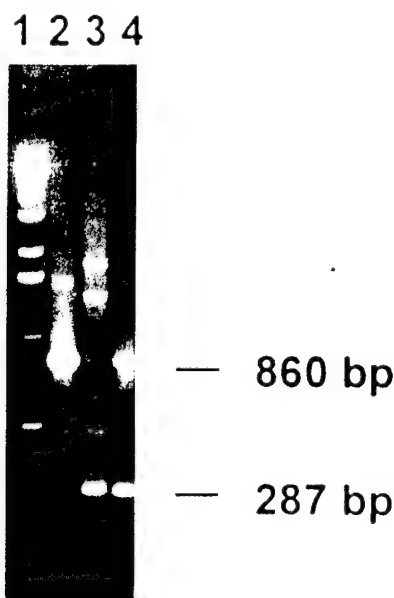


Figure 2. PCR analysis of recombinant adenoviral vector with LP-P/LacZ gene. DNA was isolated from plaques resulting from calcium phosphate transfection of the AdLPLacZ shuttle vector and the pBHG10 adenoviral vector with a deletion in the E1 gene region. The homologous recombination event would take place such that the LP/LacZ gene would be inserted into the adenoviral vector in the E1 gene region. PCR was performed on this DNA as outlined in *Materials and Methods*. The adenoviral vector strain 5 primers generate an 860-bp fragment, and the LacZ primers generate a 287-bp fragment. Lane 1, molecular weight markers; lane 2, control for the adenoviral vector; lane 3, control for the LacZ shuttle vector; lane 4, recombinant vector. We conclude that the desired vector was obtained.

nologies, Gaithersburg, Md) supplemented with 10% FBS (in the case of the HT-1080, WI-26-VA4, CCD-944SK, CCD-862SK, PA-1, BT-20, MDA-MB-468, SKOV-3, and IGROV-1 cell lines); in Dulbecco's modified Eagle's medium/F12 supplemented with 10% FBS and 10 μ g/mL of insulin (in the case of the MCF-7, MDA-MB-436, and OVCAR-3 cell lines), and in RPMI 1640 (Life Technologies) supplemented with 10% FBS (in the case of the HL-60 and U937 cell lines). All cell lines were purchased from the American Type Culture Collection (Manassas, Va).

Preparation of patient samples

Samples of ovarian cancer or normal peritoneal surface tissue were obtained by P.E.S. as incidental specimens during diagnostic and therapeutically indicated surgical procedures for the treatment of ovarian cancer. The procurement of these samples was performed as part of protocols approved by the Yale Human Investigations Committee. The tissue samples were minced with scissors, treated with disaggregation medium and collagenase, and rinsed; viable cell counts were determined by trypan blue exclusion. Cells were then immediately exposed to the vector for a 24-hour period and subsequently stained for LacZ gene activity.

Infection of cells and detection of β -gal activity

For infection and the detection of β -gal activity by staining, cells were exposed to complete tissue culture medium supple-

mented at 10% by volume with AdLPLacZ crude viral lysate of an overnight culture. After 24 hours, cells were fixed at room temperature for 10 minutes with a solution of 2% formaldehyde and 0.2% glutaraldehyde, washed three times with phosphate-buffered saline, and exposed overnight to 5-bromo-4-chloro-3-indolyl β -D-galactoside (X-Gal) (1 mg/mL) in phosphate-buffered saline containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 2 mM MgSO_4 at 37°C.¹⁵ For flow cytometric analysis and sorting (FACS) of cells for β -gal activity, cells (1×10^5 cells/well in a 24-well plate) were infected with AdLPLacZ or AdCMVLacZ at a multiplicity of infection of 25 or 50 in medium containing 2% FBS for 90 minutes. At 24 or 48 hours after the initial infection, cells were subjected to FACS-gal analysis as described by Roederer et al.¹⁵ Briefly, after harvesting by centrifugation (HL-60 and U937) or by trypsinization, cells were resuspended with 50 μ L of staining medium (RPMI 1640 supplemented with 4% FBS and 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.4) containing chloroquine and incubated for 20 minutes at 37°C. Cells were then exposed to 50 μ L of 2 mM of fluorescein di- β -D-galactopyranoside for 60 seconds, following which 1 mL of ice-cold Quench solution (staining solution with propidium iodide) was added; subsequently, cells were further incubated on ice for 1 hour. After adding phenylethyl β -D-thiogalactopyranoside, cells were subjected to FACS analysis.

RESULTS

Generation of AdLPLacZ

We have generated a recombinant adenoviral vector, AdLPLacZ, in which the *E. coli* LacZ reporter gene is placed downstream of a 2.4-kb DNA sequence (−2263 to +118 bp with respect to the transcription start site) that was taken from the human LP 5' transcriptional regulatory region (Fig 1). The E3 region and most of the E1 region were removed from this vector.⁴ The expression cassette containing the LP-P and the LacZ gene replaces the E1 region of the adenovirus strain 5. Lin et al.¹¹ have indicated that activation of the LP gene in tumorigenesis is governed by *cis*-acting elements present in 5.1 kb of DNA located in the transcriptional regulatory region immediately 5' to the LP gene. However, we incorporated a 2.4-kb DNA sequence of the LP gene (−2263/+118) in this study, largely attributable to the limited capacity of the adenoviral vector, pBHG10, in which a maximum of 7.5 kb of exogenous sequence can be inserted. PCR analysis of DNA isolated from plaque-purified clones of this vector (see Fig 2) documented the successful introduction of β -gal into these vectors. We have tested this AdLPLacZ vector in 293 cells by a staining method to determine whether this vector could produce functional β -gal enzyme. Intense blue staining was observed in 293 cells that have been infected with the AdLPLacZ crude viral lysate (data not shown).

Testing of AdLPLacZ in normal and neoplastic cells

To test the ability of the 2.4 kb of LP-P to direct the expression of the LacZ transgene in a tumor cell-specific manner, we infected various human cell lines with the AdLPLacZ crude viral lysate and monitored β -gal activ-

Table 1. Comparison of X-Gal Staining in Various Human Cell Lines After Infection with AdLP.LacZ

Cell lines	X-Gal staining
Ovarian carcinoma	
PA-1	+
OVCAR-3	+
SKOV-3	+
Hematopoietic cells	
U937	-
HL-60	-
Fibrosarcoma	
HT-1080 Simian virus-transformed	+
WI-26-VA4 Human skin fibroblast	-
CCD-944SK	-
CCD-862SK	-
Breast carcinoma	
MCF-7	+
BT-20	+
MDA-MB-436	+
MDA-MB-468	+

ity by a chrometric staining with X-Gal as described in *Materials and Methods*. The results of this experiment are shown in Table 1. Intensely staining blue color reactions catalyzed by the LP-*LacZ* adenoviral vector were produced in all of the human ovarian carcinoma cell lines tested, such as PA-1, OVCAR-3, and SKOV-3, as well as in human mammary carcinoma cell lines such as MCF-7, BT-20, MDA-MB-436, and MDA-MB-468. However, no such staining was detected in normal human skin fibroblast cell lines such as CCD-862SK and CCD-944SK, even at 3 days after initiation of the staining reaction (see Table 1). A human fibrosarcoma cell line, HT-1080, which expresses a high level of endogenous LP,¹² developed a dark-blue staining upon infection with this viral vector, whereas the cell line WI-26-VA4, which is not known to express LP from its endogenous gene,¹² exhibited no staining by this method. Because LP is constitutively expressed in normal mature hemopoietic cell lines, we assumed that the human leukemia cell lines U937 and HL-60 might express abundant LP. However, only a small percentage of cells exhibited a blue color upon exposure to AdLP.LacZ (see Fig 3 and Tables 1 and 2). This low level of LacZ reactivity may be attributable to the low infectivity of early hematopoietic cells for the adenoviral vector (see below).

To measure the transduction efficiencies of the AdLP vector and the relative strength of LP-P in cell lines, we performed FACS-gal analysis of the adenoviral vector-infected cells following exposure to the AdLP.LacZ or AdCMV.LacZ vectors at a multiplicity of infection (ratio of infectious viral particles to nucleated cells) of 50. At 24–48 hours postinfection, cells were subjected to FACS-gal analysis. In Figure 3, we present the representative results of FACS analysis of β -gal staining of the infected cells.

Less than 1% of the CCD-862SK and CCD-944SK normal human skin fibroblast cell lines expressed β -gal

Table 2. FACS-Gal Analysis

Cell lines	FACS-gal analysis	
	LP/CMV (%)	Range (LP, CMV)
Ovarian carcinoma		
OVCAR-3	0.68	(30–95, 50–95)
SKOV-3	1.45	(20–70, 10–45)
Hematopoietic cells		
U937	0.8	(1–5, 1–5)
Fibrosarcoma		
HT-1080	0.7	(20–90, 40–80)
Simian virus-transformed		
WI-26-VA-4	0.27	(5–20, 40–50)
Human skin fibroblast		
CCD-944SK	0.07	(1–2, 15–45)
CCD-862SK	0.11	(1–2, 15–30)
Breast carcinoma		
BT-20	0.23	(10, 30–50)
MDA-MB-468	0.95	(75–97, 80–97)

Data were obtained from six independent experiments. The percentage of cells expressing β -gal, determined by FACS-gal analysis, for the LP and CMV vectors, is indicated on the right side of the Table (LP, CMV). The relative activity, as measured by the ratio of the percentage of cells expressing β -gal with the LP and CMV promoters, is on the left side of the Table (LP/CMV (%)).

upon infection with AdLP.LacZ, in which the LP-P was driving the *LacZ* gene; however, $\geq 25\%$ of the cells are infectable by the adenoviral vector, as suggested by the blue color, which develops when the same cells are infected with the control vector, AdCMV.LacZ, in which the CMV promoter is driving the *LacZ* gene. These results indicated that the absence of *LacZ* expression in normal skin fibroblasts infected with the LP- β -gal adenoviral vector did not result from the low infection efficiency of AdLP.LacZ in normal fibroblasts, but rather from a low transcriptional activity of the LP-P in fibroblast cell lines.

In contrast, in a cell line known to express high levels of LP (i.e., HT-1080), nearly 90% of the cells were positive for infection by the AdLP.LacZ vector and for expression of the *LacZ* gene, as measured by positivity in the β -gal staining assay. Infecting SKOV-3, a human ovarian carcinoma cell line, with the AdLP.LacZ and AdCMV.LacZ vectors generated 70–100% and 45% positivity of cells in the β -gal assay, respectively. A high percentage of β -gal-expressing cells was observed in MDA-MB-468 cells (a human breast carcinoma cell line) following exposure to either AdLP.LacZ or AdCMV.LacZ. These results suggest that the LP-P is almost as active as the CMV promoter in ovarian and breast cancer cell lines.

In contrast, only $\sim 5\%$ of the U937 human leukemia cells were positive in the β -gal assay when these cells were exposed to either the AdLP.LacZ or the AdCMV.LacZ vector, confirming the fact that adenoviral vector infects early hematopoietic cells very inefficiently or not at all.^{16–18} These results, which were obtained from six independent experiments, are summarized in Table 2.

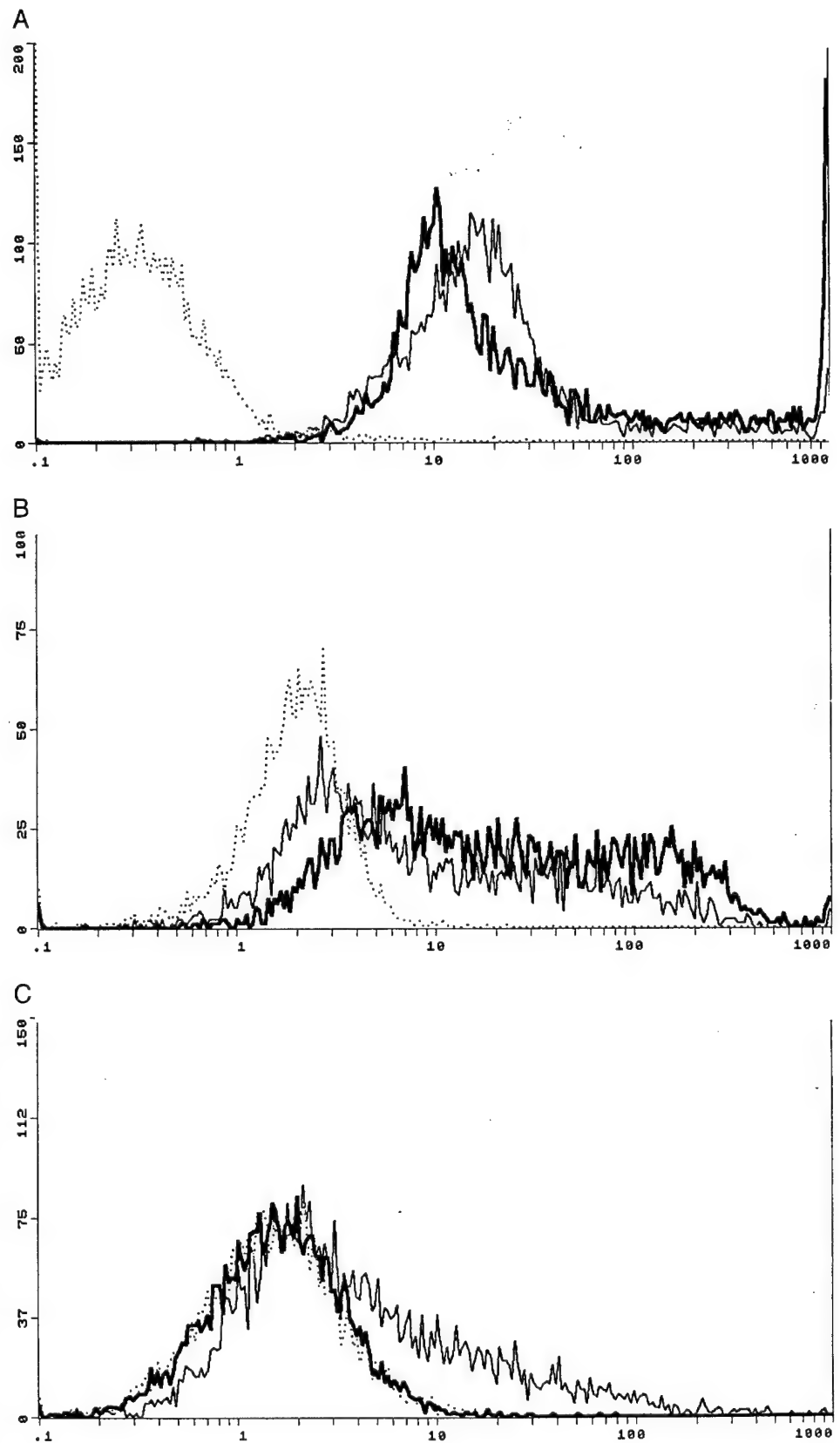


Figure 3. FACS-gal analysis of the cell lines exposed to AdLPLacZ and AdCMVLacZ adenoviral vectors. Cell lines derived from patients with: **A**, breast cancer (MDA-MD-468); **B**, ovarian cancer (SKOV-3); **C** and **D**, skin fibroblasts (CCD-862SK and CCD-944SK); **E**, hematopoietic leukemia cells (U937); and **F**, simian virus 40-transformed cells (HT-1080) were exposed to either the AdLPLacZ vector (dark solid line) or the AdCMVLacZ vector (light solid line) and compared with cells exposed to no vector (light dotted line) under the conditions analyzed in *Materials and Methods*. The cells were then stained by the X-Gal reaction and analyzed by a fluorescence detection scanner.

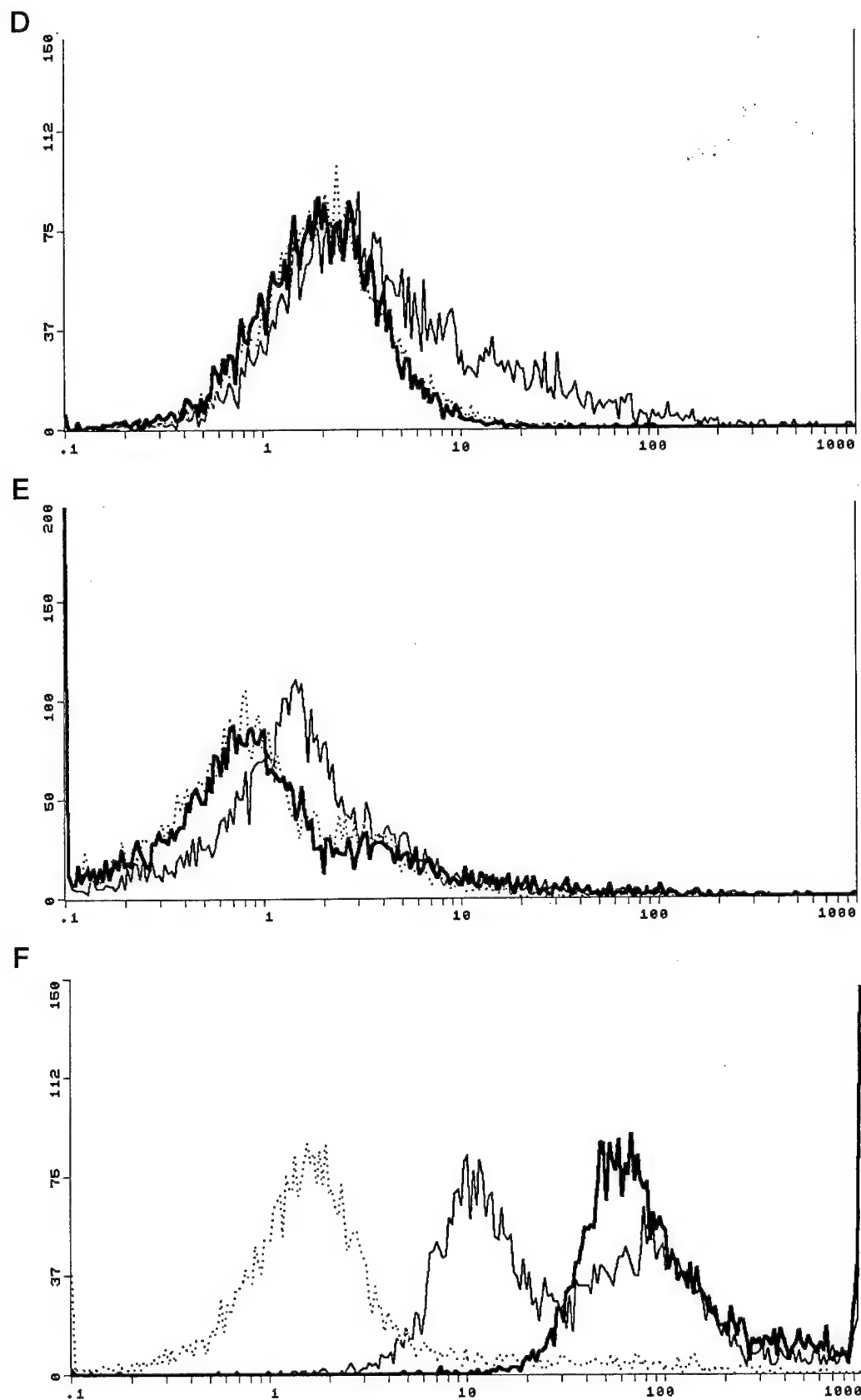


Figure 3. Continued

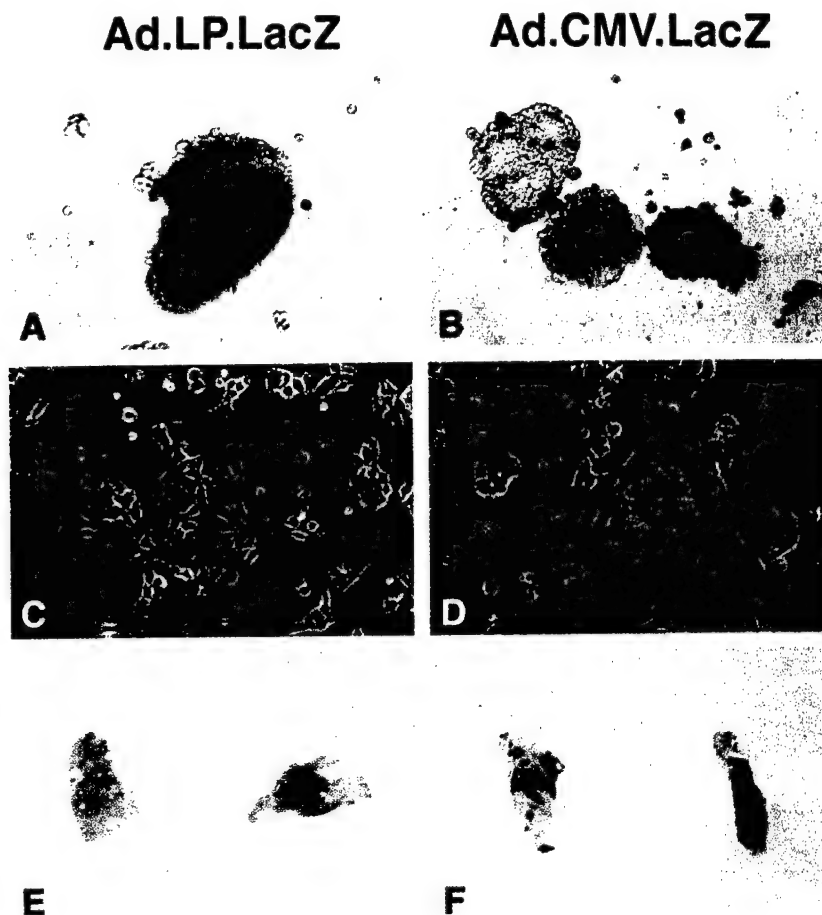


Figure 4. X-Gal staining studies of normal mesothelial cells and malignant ascites from an ovarian cancer patient. Fresh samples from patients were exposed to 0.5 mL of medium containing 150 million plaque-forming units of the adenoviral vector (except for **E** and **F**, in which 50 million plaque-forming units were used) for 90 minutes, washed, and incubated in the absence of the vector for another 24 hours; next, samples were exposed to the conditions of the X-Gal staining. Ascitic ovarian cancer cells were infected with either the AdLP.LacZ (**A** and **C**) or the AdCMV.LacZ (**B** and **D**) vectors. Biopsy cells from the mesothelium of patients undergoing surgical procedures were exposed to either the AdLP.LacZ (**E**) or AdCMV.LacZ (**F**) vectors.

The relative activities of the LP and CMV transcriptional promoters in infected cell lines, when measured by the percentage of β -gal-positive cells, are also documented by these data. In summary, the AdLP vector system could direct the expression of a heterologous gene in a neoplastic cell-dependent manner. These data suggest that the level of expression would be dependent upon the endogenous level of LP expression.

Testing of AdLP.LacZ in normal peritoneum and ascitic ovarian cancer cells from patients

To determine the susceptibility to infection by adenoviral vectors and the level of expression of transgenes driven by the LP-P in ovarian cancer cells and normal mesothelial peritoneal lining cells obtained from patients, we incubated ascitic ovarian cancer cells and a portion of a biopsy of the normal mesothelial cells of the peritoneum with either the AdLP.LacZ or the AdCMV.LacZ vectors overnight. At 2 days after infection, β -gal expression was determined by X-Gal staining. As shown in Figure 4, LacZ expression was visible in the ascitic ovarian cancer cells that had been infected with AdLP.LacZ (Fig 4, **A** and **C**) as well as with AdCMV.LacZ (Fig 4, **B** and **D**). In the portion of the peritoneal biopsy that contained normal mesothelial cells, however, the cells did not produce any blue staining upon infection

with the vector in which the LacZ gene is under the control of the LP-P (Fig 4E); cells that had been infected with a vector in which LacZ gene is driven by the CMV promoter expressed the β -gal in a dose-dependent manner (Fig 4F).

In summary, an adenovirus vector with a CMV promoter infected and expressed the LacZ gene in ascitic ovarian cancer cells as well as in normal peritoneal mesothelial cells. However, an adenoviral vector with the LP-P directing the LacZ gene was expressed in ovarian cancer cells but not in the normal mesothelial cells obtained from a single patient at the time of a therapeutically indicated surgical procedure. These studies indicated that the ascitic ovarian cancer cells and normal mesothelial cells were both infectable by the adenoviral vectors, because they both expressed the LacZ gene driven by the CMV promoter in an adenoviral vector. In contrast, the LP-P-driven LacZ adenoviral transcription unit was expressed in the ovarian cancer cells but not in the normal mesothelial cells.

DISCUSSION

Usually the extent of expression of therapeutic transgenes in cells infected by the adenoviral vectors carrying those genes in a transcription unit driven by the CMV

promoter is determined largely by infection efficiency. The sensitivity of a cell to infection by the adenoviral vector depends not only upon the number of receptors on the cell surface for the fibrillar protein, which mediates binding, but also upon the presence of the $\alpha V\beta 3$ and $\alpha V\beta 5$ integrins that bind to the adenovirus penton base proteins, thereby mediating the uptake of the adenovirus at the cell membrane level and its release from postuptake endosomes.^{19,20} As an example, epithelial cells are readily infectable by the adenoviral vector, but early hematopoietic cells, because they do not contain the requisite integrin receptors, are not readily infectable without an induction of differentiation of these cells, which increases the levels of the necessary integrins on the cell surface (see Refs. 19–21). However, in this study, we have generated an adenoviral vector system that contains 2.4 kb of LP-P regulating the expression of the *LacZ* reporter gene in neoplastic cells. In cells exposed to this vector, the expression of the transgene is governed not only by the infectability of cells by the vector but also by the activity of the LP-P in the infected cells. We have demonstrated *in vitro* that the 2.4 kb of LP-P nearest the transcription initiation site of the gene could direct heterologous gene expression preferentially in neoplastic, but not in normal, mesothelial cells. In addition, we have shown that the LP-P activity has a strength of transcriptional activity in neoplastic epithelial cells that is comparable with that seen with the CMV promoter. Thus, the AdLP vector carrying a prodrug activation chemotherapy sensitization transcription unit may have significant utility in the chemosensitization of the ovarian cancer cells present as implants on the peritoneal surface or as ascites to intraperitoneal chemotherapy, because the LP-P in the studies presented in this paper was very active in ovarian cancer cell lines and in fresh ascitic ovarian cancer cells but was not active in cell lines of mesodermal origin or in normal peritoneal mesothelial cells.

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REFERENCES

- Ferrari G, Rossini MS, Giavazzi R, et al. An *in vivo* model of somatic cell gene therapy for human severe combined immunodeficiency. *Science*. 1991;251:1363–1366.
- Hanania EG, Fu S, Roninson I, Zu Z, Deisseroth A. Resistance to taxol chemotherapy produced in mouse marrow cells by safety-modified retroviruses containing a human MDR-1 transcription unit. *Gene Ther*. 1995;2:279–284.
- Rosenfeld MA, Yoshimura K, Trapnell BC, et al. *In vivo* transfer of the human cystic fibrosis transmembrane conductance regulator gene to the airway epithelium. *Cell*. 1992;68:143–155.
- Brody SL, Jaffe HA, Han SG, Wersto RP, Crystal RG. Direct *in vivo* gene transfer and expression in malignant cells using adenovirus vectors. *Hum Gene Ther*. 1994;5:437–447.
- Hirschowitz EA, Ohwada A, Pascal WR, Russi TJ, Crystal RG. *In vivo* adenovirus-mediated gene transfer of the *Escherichia coli* cytosine deaminase gene to human colon carcinoma-derived tumors induces chemosensitivity to 5-fluorocytosine. *Hum Gene Ther*. 1995;6:1055–1063.
- Descamps V, Duffour MT, Mathieu MC, et al. Strategies for cancer gene therapy using adenoviral vectors. *J Mol Med*. 1996;74:183–189.
- Bramson JL, Graham FL, Gaudie J. The use of adenoviral vectors for gene therapy and gene transfer *in vivo*. *Curr Opin Biotechnol*. 1995;6:590–595.
- Zhang WW, Fang X, Mazur W, French BA, Georges RN, Roth JA. High-efficiency gene transfer and high-level expression of wild-type p53 in human lung cancer cells mediated by recombinant adenovirus. *Cancer Gene Ther*. 1994;1:5–13.
- Leavitt J. Discovery and characterization of two novel human cancer-related proteins using two-dimensional gel electrophoresis. *Electrophoresis*. 1994;15:345–357.
- Park T, Chen ZP, Leavitt J. Activation of the leukocyte platin gene occurs in most human cancer cells. *Cancer Res*. 1994;54:1775–1781.
- Lin CS, Park T, Chen ZP, Leavitt J. Human platin genes. *J Biol Chem*. 1993;268:2781–2792.
- Lin CS, Chen ZP, Park T, Ghosh K, Leavitt J. Characterization of the human L-platin gene promoter in normal and neoplastic cells. *J Biol Chem*. 1993;268:2793–2801.
- Zhang WW, Fang X, Branch CD, Mazur W, French BA, Roth JA. Generation and identification of recombinant adenovirus by liposome-mediated transfection and PCR analysis. *Biotechniques*. 1993;15:868–872.
- Graham FL, Prevec L. Manipulation of adenovirus vectors. In: Murray EJ, ed. *Methods in Molecular Biology*. Clifton, NJ: The Human Press; 1991:109–128.
- Roederer M, Fiering S, Herzenberg LA. FACS-gal: flow cytometric analysis and sorting of cells expressing reporter gene constructs. *Methods*. 1991;2:248–260.
- Neering SJ, Hardy SF, Minamoto D, Spratt SK, Jordan CT. Transduction of primitive human hematopoietic cells with recombinant adenovirus vectors. *Blood*. 1996;88:1147–1155.
- Clarke MF, Apel IJ, Benedict MA, et al. A recombinant bcl-x₂ adenovirus selectively induces apoptosis in cancer cells but not in normal bone marrow cells. *Proc Natl Acad Sci USA*. 1995;92:11024–11028.
- Seth P, Brinkmann U, Schwartz GH, et al. Adenovirus-mediated gene transfer to human breast tumor cells: an approach for cancer gene therapy and bone marrow purging. *Cancer Res*. 1996;56:1346–1351.
- Wickham TJ, Mathias P, Cheresh DA, Nemerow GR. Integrins $\alpha V\beta 3$ and $\alpha V\beta 5$ promote adenovirus internalization but not virus attachment. *Cell*. 1993;73:309–319.
- Wickham TJ, Filardo EJ, Cheresh DA, Nemerow GR. Integrin $\alpha V\beta 5$ selectively promotes adenovirus-mediated cell membrane permeabilization. *J Cell Biol*. 1994;127:257–264.

Appendix #2

ADENOVIRAL VECTORS WITH E1A REGULATED BY TUMOR SPECIFIC PROMOTERS ARE SELECTIVELY CYTOLYTIC FOR BREAST CANCER AND MELANOMA

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Abstract

We have previously demonstrated (Chung I, et al. (1999) *Cancer Gene Therapy* 6(2):99-106) that a truncated form of the L-plastin promoter can confer tumor specific patterns of expression on replication incompetent adenoviral vector transcription units. In this report, the L-plastin promoter was placed 5' to the E1A gene of a wild type adenovirus. The vector so generated (Ad-Lp-E1A) was directly cytotoxic to established cancer cell lines from estrogen dependent tissues and to primary explant cultures derived from ovarian cancer. This vector was not cytotoxic to cell lines in which the L-plastin E1A transcription unit was not expressed, whereas the same cell lines were sensitive to the cytotoxic effect of a replication competent adenoviral vector in which the CMV promoter drove E1A expression. When the tyrosinase promoter/enhancer was placed 5' to the E1A gene in the adenoviral backbone, the resulting vector (Ad-Tyr-E1A) was selectively toxic to melanoma cells and 100 fold less toxic to explants of ovarian cancer cells than was the Ad-Lp-E1A vector. Injection of these vectors (Ad-Lp-E1A and Ad-Tyr-E1A) into nodules derived from human breast cancer and melanoma cell lines respectively, which were growing subcutaneously in SCID mice, induced regression of these tumors. Such vectors may therefore be useful in cancer treatment.

Introduction

Recombinant adenoviral vectors have been widely used in preclinical models for in vitro and in vivo gene transfer. Adenovirus-mediated therapeutic gene expression has been achieved in a broad spectrum of eukaryotic cells, and is independent of cell replication (1,2). In addition, the E1 gene-deleted, replication-defective adenoviruses can accommodate large DNA inserts. However, the limitations of this vector system for cancer gene therapy have included nonselective delivery of therapeutic genes to both normal cells and tumor cells, and by their inability to spread to neighboring tumor cells following infection of tumor cells (3). One of the strategies used to circumvent these limitations have included the use of tissue specific promoters or enhancers that are active in tumor tissue and the corresponding normal tissue to direct the replication of the adenovirus in the desired target cells. In this context, the minimal promoter/enhancer from the prostate-specific antigen (PSA) gene has been used to drive E1A expression and thereby create an adenovirus, designated CN706, that selectively replicates in PSA-positive cells (4). A similar strategy using the albumin promoter has been used to develop a herpes simplex virus that selectively replicates in hepatoma cells (5). These vectors are directly toxic to the tumor cells in which they selectively replicate, and are toxic to the corresponding normal tissue.

In our laboratory, we have been studying the feasibility of using a truncated form of the L-plastin promoter to create adenoviral vectors which selectively replicate within tumor cells and therefore are selectively toxic to these cells. The plastins constitute a family of human actin-binding proteins (isoforms) which are abundantly expressed in all normal replicating mammalian cells. One isoform, L-plastin, is constitutively expressed at high levels in mature hematopoietic cell types, but is expressed in no other normal tissue. L-plastin is, however, constitutively synthesized in most types of malignant human cells, suggesting that its expression is induced during tumorigenesis. L-plastin expression is especially high in cancers which arise from estrogen-dependent tissues (6,7,8,9). In order to test the feasibility of conferring tumor specific conditional replication competency on the wild type adenovirus, we have placed a 2.5 Kb truncated form of the

L-plastin promoter 5' to the E1A gene. For comparison, we have also placed the tyrosinase promoter/enhancer 5' to the E1A gene in a wild type adenovirus. Tyrosinase, which is the product of the albino locus and is a pigment cell-restricted enzyme that catalyzes the rate limiting step in melanin synthesis, is highly expressed in melanoma cells. The tyrosinase promoter/enhancer cassette used for this purpose has been studied in human and mouse cells (10,11,12) for its ability to govern the expression of heterologous genes in the adenoviral vector.

The Ad-Lp-E1A vector is toxic to L-plastin positive breast cell lines as well ovarian cancer cells in vitro and when injected intratumorally, suppresses the growth of L-plastin-positive breast cancer cells in SCID mice. We also demonstrate that the vector which contains the tyrosinase promoter/enhancer cassette driving the E1A gene (Ad-Tyr-E1A) suppresses the growth of a melanoma cell line in SCID mice. The results demonstrate that the Ad-Lp-E1A vector in which the E1A gene expression is regulated by a truncated L-plastin promoter is 100 times as toxic to ovarian cancer cells than is the Ad-Tyr-E1A vector, which itself is toxic to melanoma cell lines. Our results also suggested that a correlation exists between the ability of cell lines to support the expression of the E1A gene following exposure to the Ad-Lp-E1A vector, and the degree of rounding up and lysis of the exposed cells.

Material and Methods

Cells and Cell Culture. The following cells were obtained from the American Type Culture Collection (Manassas, Virginia, USA): The MCF-7 and MDA-MB-231 human breast cancer cell lines; The Ovar-3 human ovarian cancer cell line; The CCD fibroblast cell line and the human embryonic kidney (HEK) 293 cell line. The TF-2 and Yusac-2 human melanoma cell lines were obtained from Dr. Ruth Halaban at Yale University. All cells were cultured in DMEM medium supplemented with 10% heat-inactivated fetal bovine serum (FBS).

Structure of the L-plastin and Tyrosinase Promoter E1A Replication-Competent Adenoviral Vectors. A DNA fragment which contains the E1 gene was generated from wild type adenovirus 5 using the following PCR primers: 5' acgcgtcgacgcgagtagagttttctcctccg 3' and 5' agcttggttaactcgaggacaggcctctcaag 3' (GeneAmp XLPCR kit, Perkin Elmer). The 5,200bp PCR fragment was digested with SalI and PmeI restriction enzymes and then ligated into the pShuttle plasmid (13) which has been cut with PmeI and SalI to produce a new shuttle plasmid (pADshuttle) which contains a complete E1 gene but a deleted E1A promoter. Then the promoter fragments were inserted between the NotI and SalI site of the pADshuttle (Fig. 1). The L-plastin ScaI promoter was inserted into pBluescript Sk+ after it was excised from AdLpLaz (6). The L-plastin ScaI promoter fragment was then cut from the Pbluescript Sk+ with NotI and XhoI. It was then ligated into the pAD shuttle plasmid. The human tyrosinase promoter-enhancer was synthesized by PCR from human Yusac-2 genome with three pairs of primers: HTP1 5'ccggaattcattctaaccataagaattaa3', HTP2 5'acgcgtcgacggaactggctaattggagtc3'; TE1 5' atttgcgccgcaattctgtcttcgagaacat 3', TE2 5'cgcgatccatggaaatgctgcctctg 3'; HEN1 5' cgcgatccaattctgtcttcgagaacat 3' HEN2 5'ccggaattcatggaaatgctgcctctg 3'. The HTP fragment was cut with Sal I and EcoR I. The HEN fragment was cut with EcoR I and BamH I. The TE fragment was cut with BamH I and Not I. All three fragments were then ligated into the pBluescript SK NotI and SalI fragment. The vectors thereby generated were sequenced. After sequencing, this Not I and Sal I fragment was inserted into the pAD shuttle vector. We also synthesized the CMV promoter with PCR and inserted it into the pAD shuttle plasmid.

The replication-competent adenoviral vectors under the control of the L-plastin, tyrosinase and CMV promoters were prepared by standard homologous recombination techniques using the pAD shuttle plasmids with AdEasy-1 (kindly provided by Dr. He and Dr. B Vogelstein (13) of the Howard Hughes Medical Institute, Johns Hopkins Oncology Center) in Bj5138. After cutting with *PacI*, the adenovirus DNA plasmids, which contain different promoters, were transfected into HEK 293 cells. Each recombinant adenoviral vector was isolated from a single plaque and expanded in HEK 293 cells. Viral DNA was treated and analyzed by PCR to confirm the structure of the E1A promoters in the virus.

The vector for experimentation was prepared by infecting thirty 15-cm tissue culture plates of HEK 293 cells and then harvesting the detached cells after 48 hours. The viral particles remained associated with the cells. Cells were collected by centrifugation at 400 g for 5 minutes at 4°C. The cells were resuspended in 10 mL of cold PBS (free of Ca²⁺ and Mg²⁺), and were lysed with three cycles of freezing and thawing. The cells were collected by centrifugation at 1,500 g for 10 minutes at 4°C. The supernatant was placed on a gradient prepared with equal parts of Cesium Chloride in phosphate buffered saline (PBS) (1.45 g/mL and 1.20 g/mL), and then centrifuged for 3 hours at 15,000 g at 12°C. The virus band was removed, rebanded in a preformed Cesium Chloride gradient by ultracentrifugation for 18 hours, and dialyzed against cold PBS (pH 7.4) containing 10 mM MgCl₂ and 10% glycerol. Titers of purified adenoviral vectors were determined by spectrophotometer and by plaque assays.

Western Blot Analysis of Adenovirus E1A Protein Expression. 24 hours after viral infection, cells were lysed in cell lysis buffer (50 mM HEPES at pH 7.5, 0.15 M NaCl, 1 mM EDTA, 1% NP-40, 1 mM PMSF, 10 mM NaF, 10 ng/mL aprotinin, 10 ng/mL leupeptin, 1 mM DTT, and 1 mM sodium vanadate), incubated for 60 minutes on ice, and centrifuged at 1,500 g for 10 minutes at 4°C. The supernatants were transferred to Eppendorf tubes and were kept at 100°C for 5 minutes. Protein was analyzed by immunoblotting with monoclonal antibody (mcAb) the Ad5 E1A protein. Reactivity was visualized by enhanced chemiluminescence (Amersham Life Sciences Inc., Arlington Heights, Illinois, USA).

In Vitro Viral Replication Assay. Monolayer cell cultures in 6-well dishes (1x 10⁵ cells/well) were infected with Ad-Lp-E1A, Ad-Tyr-E1A, adenoviral

vector with the CMV promoter driving the E1A gene (Ad-CMV-E1A), the control adenoviral vector in which the CMV promoter drives the LacZ gene (Ad-CMV-LacZ) or wild-type adenovirus (Ad5) at a MOI of 10 plaque-forming units (pfu) per well. The virus was removed 2 hours after inoculation. The cells were then washed twice with PBS and incubated at 37°C for varying periods of time. Lysates were prepared with three cycles of freezing and thawing. Serial dilutions of the lysates were titrated on HEK 293 cells.

Cytopathic Effect Assays. Cells were passaged 24 hours before infection with adenoviruses at the indicated MOI. Photomicrographs were taken on days 3, 5, and 9 after infection.

In Vivo Gene Transfer to Human Breast Cancer Xenografts. Subcutaneous tumor nodules were established by subcutaneous injection of 10 million MCF-7 breast cancer cells or TF-2 melanoma cells suspended in 0.1 mL of PBS into the flanks of Female athymic nude mice or SCID mice aged 5–6 weeks. Tumor nodules were allowed to grow subcutaneously to approximately 6–7 mm in diameter. For intratumoral injection of the viral vectors, 50 μ L of viral particles suspended in PBS were injected using a 25-gauge needle. Tumor size was measured at the indicated times after injection in their longest dimension and the dimension at 90 degrees to that measurement. Tumor volumes were calculated using the following formula: $(\text{length} \times \text{width}^2)/2$. Tumor volumes were normalized to 100% on day 0 (V/V_0). Results are expressed as the fractional tumor volume (mean \pm SD) at each time point compared to that on the day of injection. Statistically significant differences were assessed with the Student's t test.

Results

Construction of Conditionally Replication Competent Adenoviral Vectors. In order to construct replication competent adenoviral vectors, a pShuttle plasmid (pADShuttle which is presented in Figure 1) was produced by replacing the right arm of the shuttle vector with a 5.2kb fragment which contains adenovirus sequence from the wild type sequence extending from the 513 nucleotide position to the 5,790 nucleotide position. We then inserted the L-plastin promoter ScaI fragment, the CMV Promoter or the Tyrosinase promoter/enhancer 5' to the E1A gene of the Adenovirus (see Figure 1). After adenoviral vector particles were generated in HEK 293 cells, the vector DNA was purified. PCR was then used to check the presence and intactness of the structure of the promoter in each adenoviral vector.

Western Blot of E1A Expression. Ad5 E1A protein expression in MDA-MB-468 breast cancer cells and in the CCD fibroblast cells following infection with Ad-Lp-E1A, Ad-Tyr-E1A or wild type adenovirus 5 was determined by Western blot analysis (see Figure 2). Wild type adenovirus 5 produced considerable E1A protein in CCD and MDA-MB-468 cells (see Figure 2, lanes 4 and 5 respectively). Ad-Tyr-E1A did not produce detectable levels of E1A protein in either the MDA-MB-468 (lane 1 of Figure 2) or CCD (lane 2 of Figure 2) cells. Ad-Lp-E1A produced a high level of E1A in MDA-MB-468 (lane 3 of Figure 2) but not in CCD (lane 6 of Figure 2) cells. Previously published experiments with replication incompetent adenoviral vectors carrying the LacZ gene driven either by L-plastin or CMV promoters showed that exposure of the CCD cell line to the Lp-LacZ vector produced no detectable expression of LacZ but exposure to the CMV-LacZ vector produced high levels of LacZ expression in CCD cells (6). This data shows that the L-plastin promoter can drive the expression of the E1A gene in breast cancer cells but not in the CCD fibroblast cells. In addition, the tyrosinase promoter/enhancer was not active in either the cell lines derived from estrogen dependent tissues or in the CCD fibroblast cell line.

Cytolysis Assays in Cell Lines and Explant Cultures of Tumor Cells Following Exposure to Viral Vectors. To examine the replication specificity of the Ad-Lp-E1A and Ad-Tyr-E1A vectors, established human

cancer cell lines and primary cultures of tumor cells were analyzed for their ability to generate rounding up and lifting off of the cells, suggesting a direct cytotoxic action of the vector and therefore replication competency in these cells. The MDA-MB-486 breast cancer cell line, which has been shown to support the expression of genes regulated by the L-plastin promoter (6, 15), and supported the expression of the E1A gene when exposed to the Ad-Lp-E1A vector (see Figure 2, lane 3), was used to test the lytic effect of the Ad-Lp-E1A vector in vitro. The breast cancer MDA-MB-468 cells were infected with Ad-Lp-E1A at different MOI. Lysis of the cells was observed at 9 days after infection at MOI 10 (see Figure 3). Another breast cancer cell line MCF-7 produced the same result (data not shown).

The CCD minimal deviation fibroblast cell line, which did not show expression of the E1A gene following exposure to the Ad-Lp-E1A vector (see lane 6 of Figure 2), but showed E1A expression following exposure to wild type adenovirus (lane 4 of Figure 2), was infected with the Ad-Lp-E1A, Ad-CMV-E1A, Ad-Tyr-E1A vectors and wild type adenovirus at MOI 100. A lytic effect was observed following exposure of the CCD cells to the wild type adenovirus and to the Ad-CMV-E1A vector after 9 days (see Figure 4). In this experiment, no lysis was seen following exposure of the CCD to the Ad-Lp-E1A nor to the Ad-Tyr-E1A vectors (see Figure 4). Neither the Ad-Lp-E1A nor the Ad-Tyr-E1A vectors produced convincing levels of expression of the E1A gene in the CCD cell line (see lanes 6 and 2 respectively of Figure 2). Thus, the results in Figures 2-4 show that there is a correlation between the expression of the E1A gene and the lysis of the cell lines following exposure to the Ad-Lp-E1A vector. These results suggest that Ad-Lp-E1A vector can replicate only in cell lines which support the expression of genes regulated by the L-plastin promoter.

In order to test for the replication competency of the Ad-Lp-E1A vector in primary or explant cultures of cancer cells, explants of primary ovarian carcinoma cells were cultured as monolayers and then exposed to the Ad-Lp-E1A vector. At MOI 1 and 10, a remarkable lytic effect was observed in the primary explants of ovarian carcinoma (see Figure 5). Then, the effect of the Ad-Lp-E1A, Ad-Tyr-E1A, Ad-CMV-E1A vectors, and the negative control Ad-CMV-LacZ adenoviral vector on explants of ovarian carcinoma cells from a patient sample was tested at different MOI (see Figure 6). The difference in the IC₅₀ between the Ad-Lp-E1A and Ad-Tyr-E1A vectors is about 100 fold (see Figure 6) and between the Ad-Lp-E1A and Ad-CMV-LacZ vectors was 1000 fold. In addition, the data in this figure shows that

the cytolytic effect of the Ad-Lp-E1A vector is equivalent to the Ad-CMV-E1A vector.

When we infected the melanoma TF-2 cell line with the Ad-Lp-E1A, Ad-Tyr-E1A, and the Ad-CMV-E1A vectors, as well as the wild type adenovirus, the cultures exposed to the Ad-CMV-E1A and Ad-Tyr-E1A vectors, as well as the wild type Adenovirus 5, show lytic effects. In contrast, cultures of the TF-2 melanoma cell line exposed to the Ad-Lp-E1A vector did not show detectable cytotoxicity (see Figure 7). This data shows that the toxic effect of the Ad-Tyr-E1A and the Ad-Lp-E1A vectors is specific to melanoma cell lines or to estrogen dependent cell lines respectively.

Treatment of A Human Breast Cancer Xenograft with the Ad-Lp-E1A Vector and A Human Melanoma Xenograft with the Ad-Tyr-E1A Vector. To evaluate the therapeutic efficacy of the Ad-Lp-E1A vector in vivo, MCF-7 breast cancer cells were injected subcutaneously in SCID mice, allowed to grow into subcutaneous tumor nodules at least 50 mm³ in size, and then injected three times with 1×10^8 pfu of Ad-Lp-E1 or Adv-CMV-LacZ, once every two days. Injection with Ad-Lp-E1 vector was associated with inhibition of MCF-7 tumor cell growth (see Figures 8A and 8B). The degree of inhibition seen following intratumoral injection of the Ad-Lp-E1A vector was equivalent at day 25 with the degree of suppression seen with the wild type adenovirus (see Figure 8B). These findings were in contrast to the progressive growth of tumors injected with the Ad-CMV-LacZ vector or with PBS (see Figure 8). These results indicate that injection of Ad-Lp-E1A can suppress the growth of the MCF-7 cell line in vivo. However, all injected cell lines eventually regrew in this model.

In a similar manner, the human melanoma TF-2 cell line was injected subcutaneously and allowed to grow into a tumor nodule in SCID mice in order to test the lytic effect of the Ad-Tyr-E1A vector on human melanoma cells in vivo. Then, these nodules were injected intratumorally once with 1×10^8 of the Ad-Tyr-E1A vector particles. As shown in Figure 9, the injection of the vector into the TF-2 tumor nodules with the wild type adenovirus or the Ad-Tyr-E1A vector resulted in a reduction in the size of the melanoma tumor nodule, whereas, no reduction in tumor growth occurred with injection of either PBS or the Ad-CMV-LacZ vector. Longer follow-up showed that the vector injected cell lines eventually regrew.

Discussion

The goal of this work was to create adenoviral vectors which displayed tumor specific replication competency which was directly cytotoxic to tumor cells. We chose to compare the cytotoxic action of replication competent vectors, which contained the L-plastin promoter 5' to the E1A gene (Ad-Lp-E1A) with another vector which contained the Tyrosinase promoter 5' to the E1A gene (Ad-Tyr-E1A). These experiments extended previous work in our laboratory with replication incompetent adenoviral viral vectors which showed that a truncated form of the L-plastin promoter was active in established neoplastic cell lines derived from estrogen dependent tissues even after being embedded in the adenoviral backbone (6, 14). We showed first that the Lp-E1A transcription unit of the Ad-Lp-E1A vector was expressed in a breast cancer cell line but not in the CCD minimal deviation fibroblast cell line. We showed as well that the Tyrosinase-E1A transcription unit was not active in breast cancer cell lines nor in the CCD cell line. We then showed that the Ad-Lp-E1A was cytotoxic to tumor cells derived from estrogen dependent tissues but not toxic to the CCD minimal deviation fibroblast cell line. This pattern of toxicity (Figures 3-5) mirrored the expression of the E1A transcription unit when placed under control of the L-plastin promoter (see Figure 2).

The Ad-Tyr-E1A vector was directly cytotoxic in vitro predominantly to tumor cell lines which corresponded to the origin of the tyrosinase promoter (melanoma cells) while being much less toxic to the breast and ovarian cancer cells. The Ad-Lp-E1A vector was much more toxic to breast and ovarian cancer cells than to melanoma. Thus, the L-plastin and tyrosinase driven E1A vectors were selective to tumor tissue in their cytotoxicity.

We were also able to show that this tumor specific cytolytic effect extended into the in vivo setting by injecting the vectors into the subcutaneous nodules of either breast cancer cells or melanoma cells in a SCID mouse xenograft. These experiments showed that the Ad-Lp-E1A vector could transiently suppress the growth of the MCF-7 vector and suppress those cells even after they had grown into an established tumor nodule. In addition, the Ad-Tyr-E1A vector transiently suppressed the in vivo growth of the TF-2 melanoma cell line in the SCID mouse model. Since the injection of the Ad-CMV-LacZ control replication incompetent adenoviral vector into the subcutaneous tumor nodules in the SCID mice did not

suppress the growth of the tumor nodules, the suppressive effect of the Ad-Lp-E1A and the Ad-Tyr-E1A vectors was most likely dependent on the replication competency of these two vectors. In addition, the lytic effect of the Ad-Lp-E1A vector on cell lines in vitro was equivalent to the effect of the Ad-CMV-E1A on the tumor nodules in the SCID mice.

Thus, the data suggest that the Ad-Lp-E1A and Ad-Tyr-E1A vectors may ultimately be of value for the development of therapeutic vectors for the treatment of solid tumor malignancies. However, the suppressive effect of the vectors was not durable. The obvious steps to undertake to make these vectors truly useful in the clinic is to add a therapeutic transcription unit so that their toxic action on tumor cells is more robust, and to engineer them such that they will bind only to tumor cells, but not to normal cells. When these goals are achieved, such vectors may be of ready for clinical testing in human patients.

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References:

1. Haj-Ahmad, Y., and Graham, F. (1986). Characterization of an adenovirus type 5 mutant carrying embedded inverted terminal repeats. *Virology*. 153:22-34.
2. Bett, A., Haddara, W., Prevec, L., and Graham, F. (1994). An efficient and flexible system for construction of adenovirus vectors with insertions or deletions in early regions 1 and 3. *Proc. Natl. Acad. Sci. USA*. 91:8802-8806
3. Heise, C., and Kim, D.H. (2000). Replication-selective adenoviruses as oncolytic agents. *J. Clin. Invest.* 105:847-851
4. Rodriguez, R. et al. (1997). Prostate attenuated replication competent adenovirus (ARCA) CN706: a selective cytotoxic for prostate-specific antigen-positive prostate cancer cells. *Cancer Res.* 57:2559-2563.
5. Miyatake, S.-I. et al. (1999). Hepatoma-specific antitumor activity of an albumin enhancer/promoter regulated herpes simplex virus in vivo. *Gene Ther.* 6:564-572.
6. Chung I, et al. (1999). Use of L-plastin promoter to develop an adenoviral system that confers transgene expression in ovarian cancer cells but not in normal mesothelial cells. *Cancer Gene Ther.* Mar-Apr;6(2):99-106.
7. Lin Cs, et al. (1997). The murine L-plastin gene promoter: identification and comparison with the human L-plastin gene promoter. *DNA Cell Biol.* 16(1):9-16.
8. Lin CS, et al. (1993). Characterization of the human L-plastin gene promoter in normal and neoplastic cells. *J Biol Chem.* (1993) Feb 5;268(4):2793-801.
9. Lin CS, et al. (1993). Human plastin genes. Comparative gene structure, chromosome location, and differential expression in normal and neoplastic cells. *J Biol Chem.* 1993 Feb 5;268(4):2781-92.
10. Siders WM, et al. (1998) Melanoma-specific cytotoxicity induced by a tyrosinase promoter-enhancer/herpes simplex virus thymidine kinase adenovirus. *Cancer Gene Ther.* Sep-Oct;5(5):281-91.
11. Siders WM, et al. (1998) T cell- and NK cell-independent inhibition of hepatic metastases by systemic administration of an IL-12-expressing recombinant adenovirus. *J Immunol.* Jun 1;160(11):5465-74.

12. Siders WM, Fenton RG, et al.(1996). Transcriptional targeting of recombinant adenoviruses to human and murine melanoma cells. *Cancer Res.* Dec 15;56(24):5638-46.
13. He TC, et al. (1998). A simplified system for generating recombinant adenoviruses. *Proc Natl Acad Sci U S A.* Mar 3;95(5):2509-14.
- Peng XY, Won JH, Rutherford T, Fujii T, Zeltermann D, Sapi E, Pizzorno G, Leavitt J, Kacinski B, Crystal R, Schwartz P, and Deisseroth A. The use of the L-plastin promoter for adenoviral mediated tumor-specific gene expression in ovarian and bladder cancer cell lines. Submitted, 2001.

Figure Legends:

Figure 1: Construction of Conditionally Replication Competent Adenoviral Vectors.

Figure 2: Western Blot of E1A Expression. Ad5E1A protein expression in MDA-MB-468 cells and CCD fibroblast cells following injection with Ad-Tyr-E1A (lanes 1,2), Ad-Lp-E1A (lanes 3,6), or wildtype adenovirus 5 (lanes 4,5) was determined by Western blot analysis. Lanes 1,3,5 contain protein extracts from MDA-MB-468 cells. Lanes 2,4,6 contain protein extracts from CCD cells.

Figure 3: Study of the Sensitivity of Breast Cancer Cells to the Ad-Lp-E1A Vector. The MDA-MB-468 breast cancer cell line was exposed to the Ad-Lp-E1A vector at 100 MOI (left hand panel), 10 MOI (middle panel), and 0 MOI (right hand panel).

Figure 4: Study of the Cytolytic Effect of the Ad-Lp-E1A Vector to the CCD Cell Line at 9 Days After Infection with Adenoviral Vectors at 100 MOI.

Figure 5: Study of the Sensitivity of Primary Cultures of Ovarian Cancer Cells to the Ad-Lp-E1A Vector at 9 Days after Infection.

Figure 6: Study of the Sensitivity of Primary Explants of Ovarian Cancer Cells to Adenoviral Vectors: Primary monolayer explants of ovarian cancer cells were exposed to varying MOI of the Ad-Lp-E1A vector, shown by the solid squares, the Ad-CMV-E1A vector, shown by the solid diamonds, the Ad-Tyr-E1A vectors shown by the open triangles, and the replication incompetent Ad-CMV-LacZ negative control vector shown by the line connected by the X's.

Figure 7: Cytotoxicity of the Adenoviral Vectors to the Melanoma TF-2 Cells at 9 Days after Infection.

Figure 8A: Photograph of Mice Either Injected with the Ad-Lp-E1A Vector or the Ad-CMV-LacZ Vector Following Injection of MCF-7 Breast Cancer Cells Subcutaneously.

Figure 8B: Plot of the Growth of the MCF-7 Cells Following Subcutaneous Injection of 100 million pfu of the Ad-Lp-E1A, and the Ad-CMV-LacZ vectors, PBS or the wild type adenovirus.

Figure 9: Growth of the TF-2 Melanoma Cell Line Subcutaneously Following Injection of 100 million pfu of the Ad-Tyr-E1A or Ad-CMV-LacZ vectors, PBS, or the wild type adenovirus.

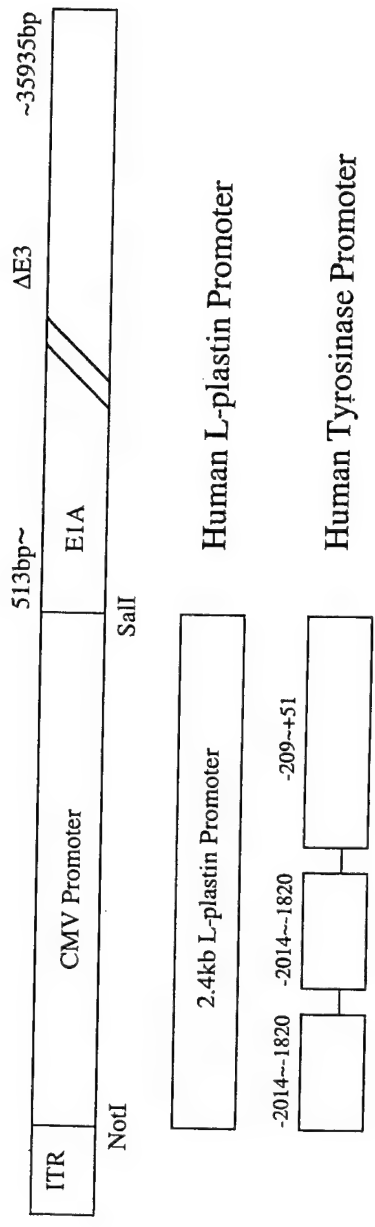
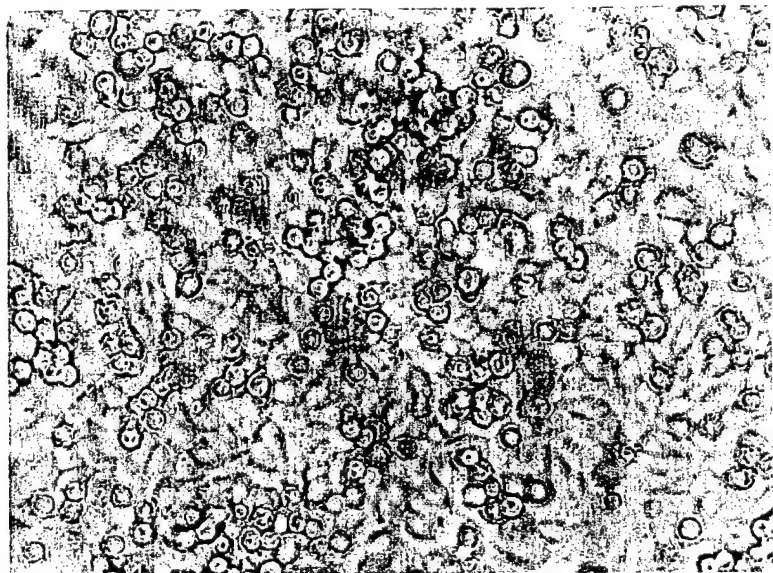


Figure 1

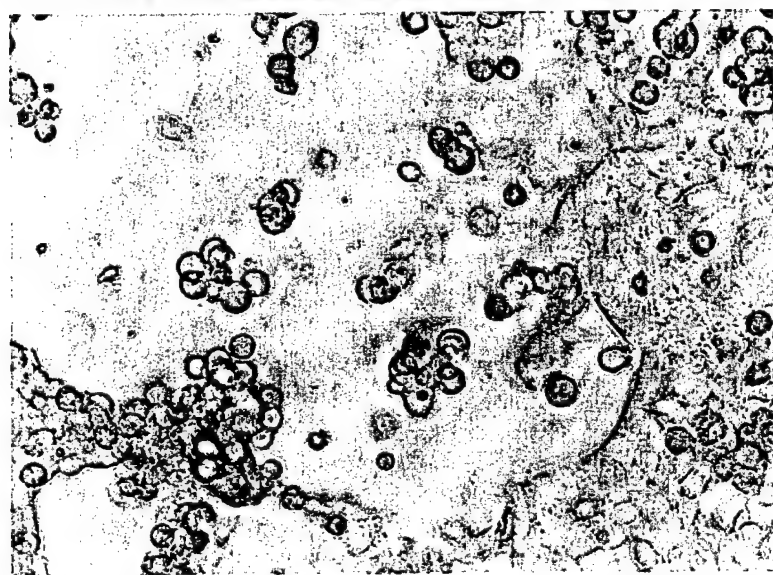


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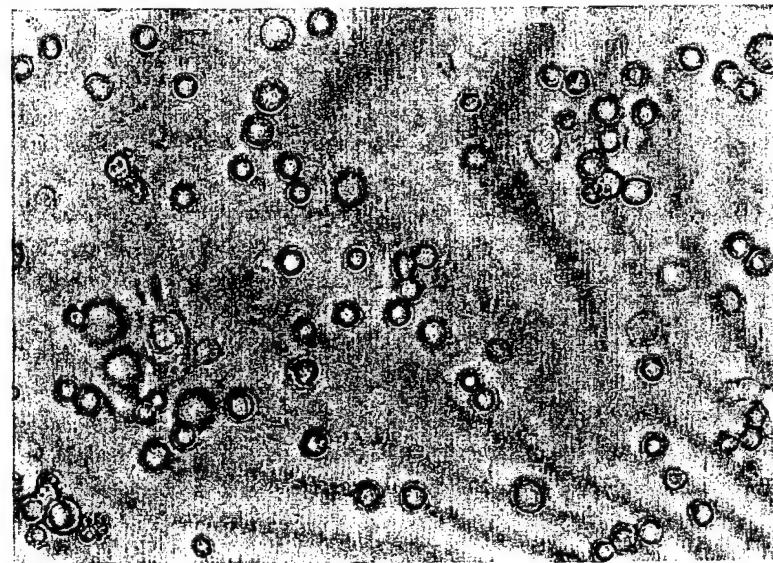
Figure 2



MOI 1



MOI 10



MOI 100

Figure 3



Figure 4

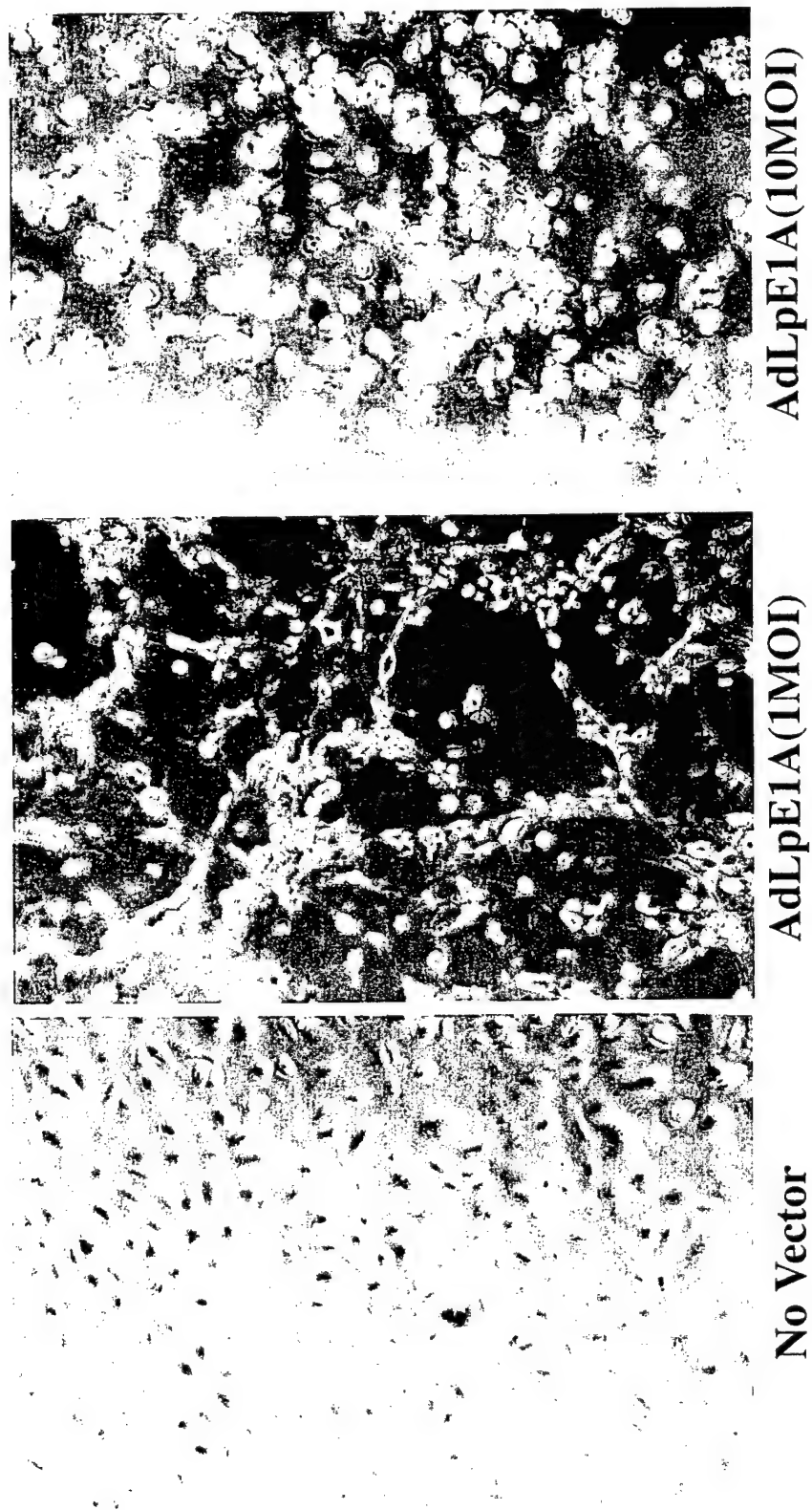
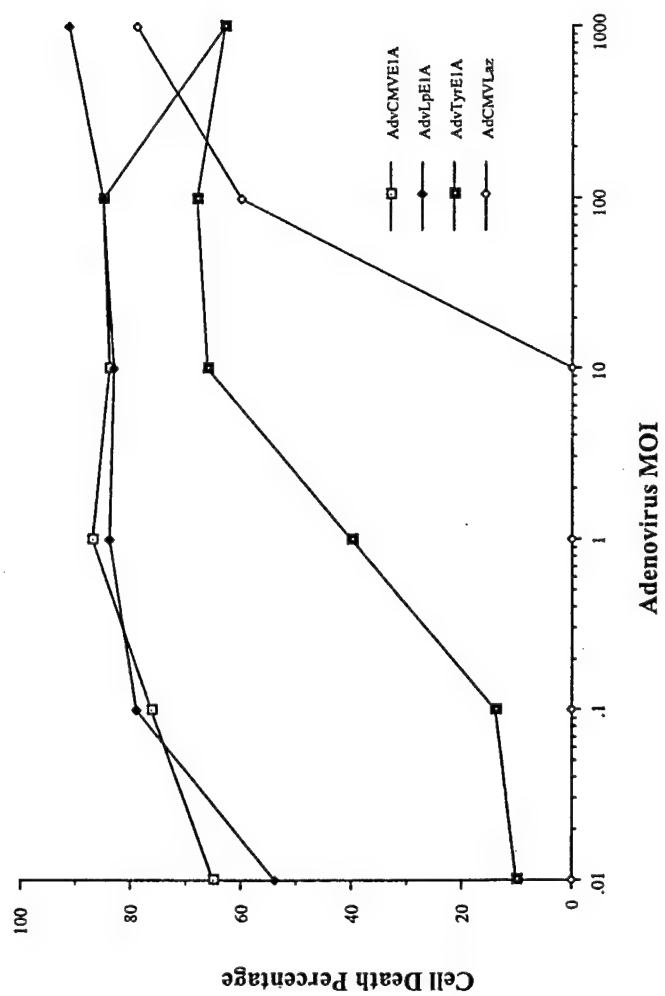


Figure 5

Figure 6



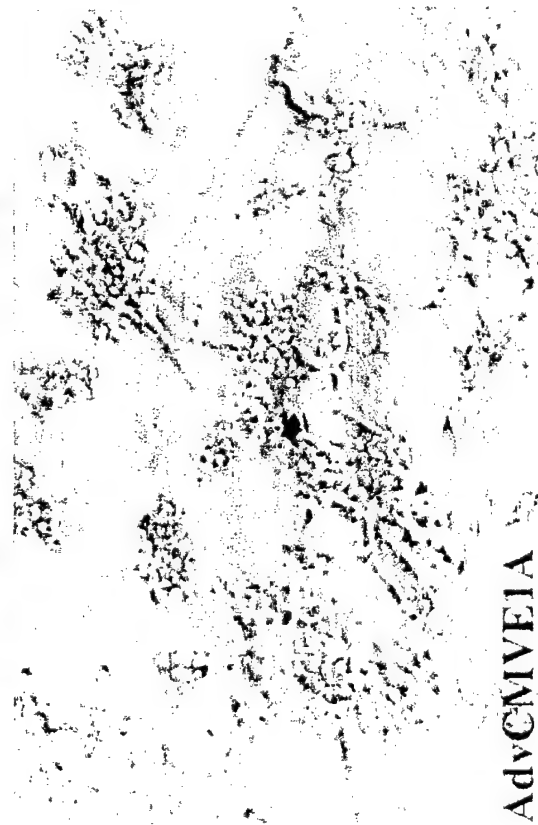
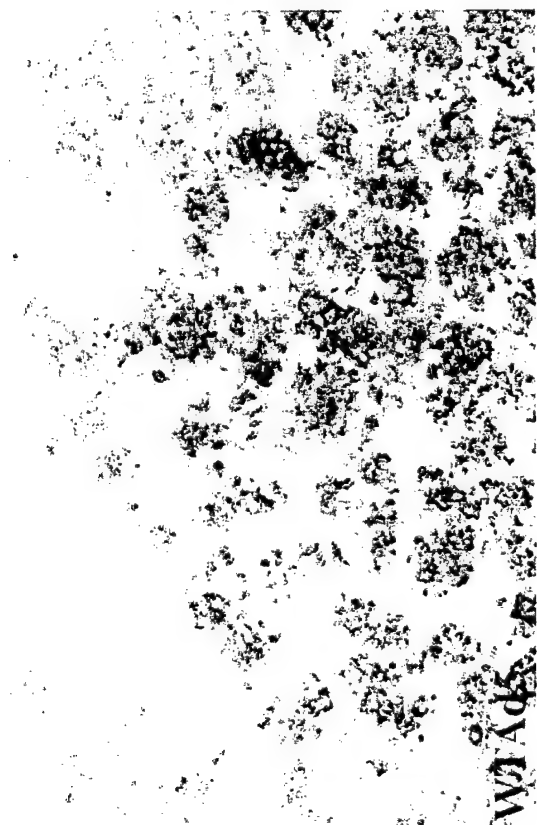
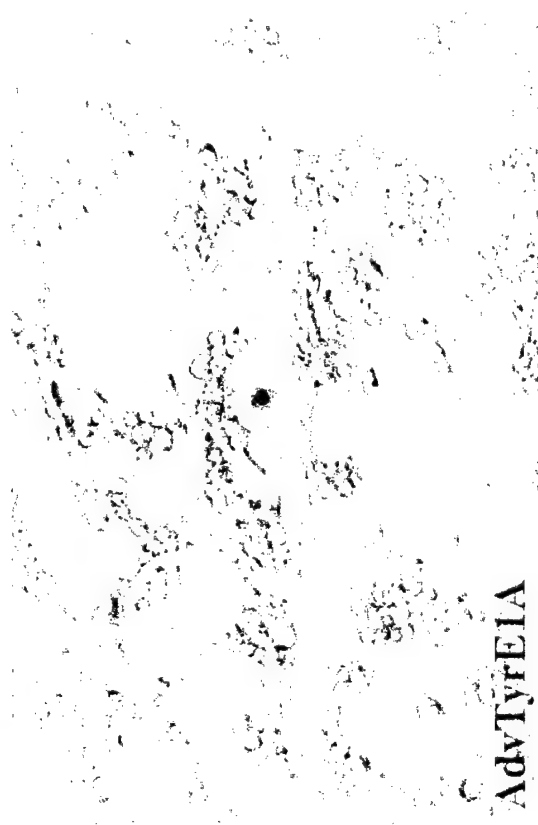
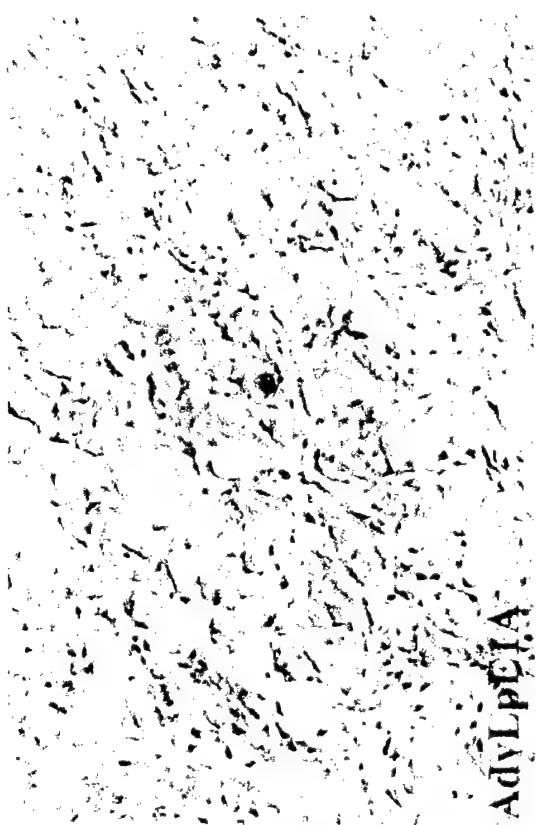


Figure 7



Figure 8A

**Replication Competent Adenovirus Suppresses
Growth of MCF-7 Cell Line in SCID mice**

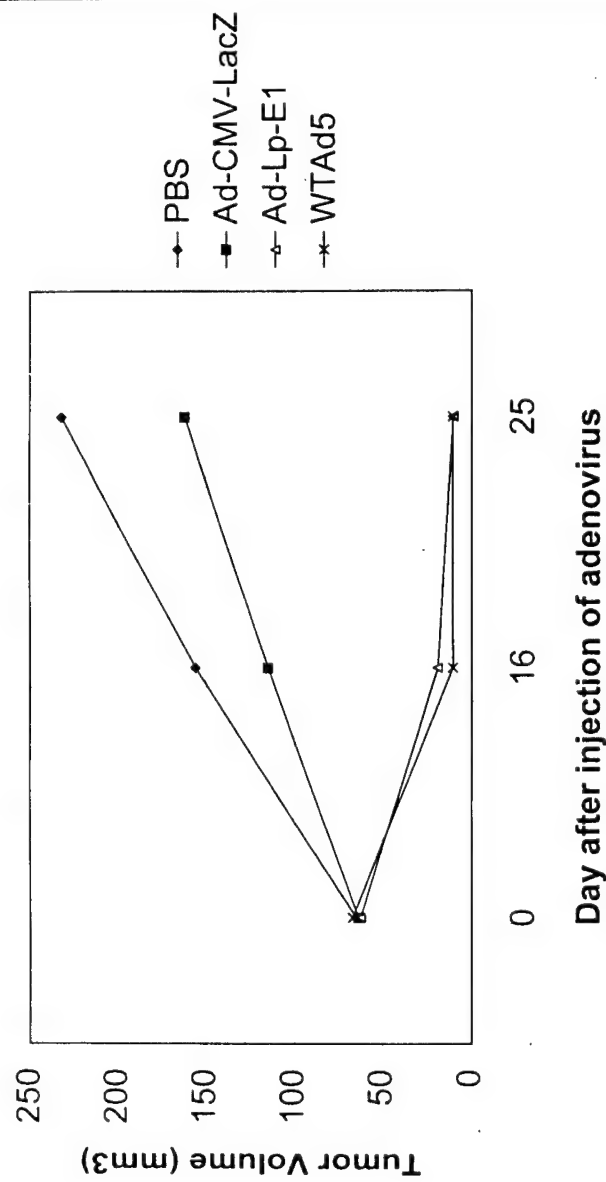


Figure 8B

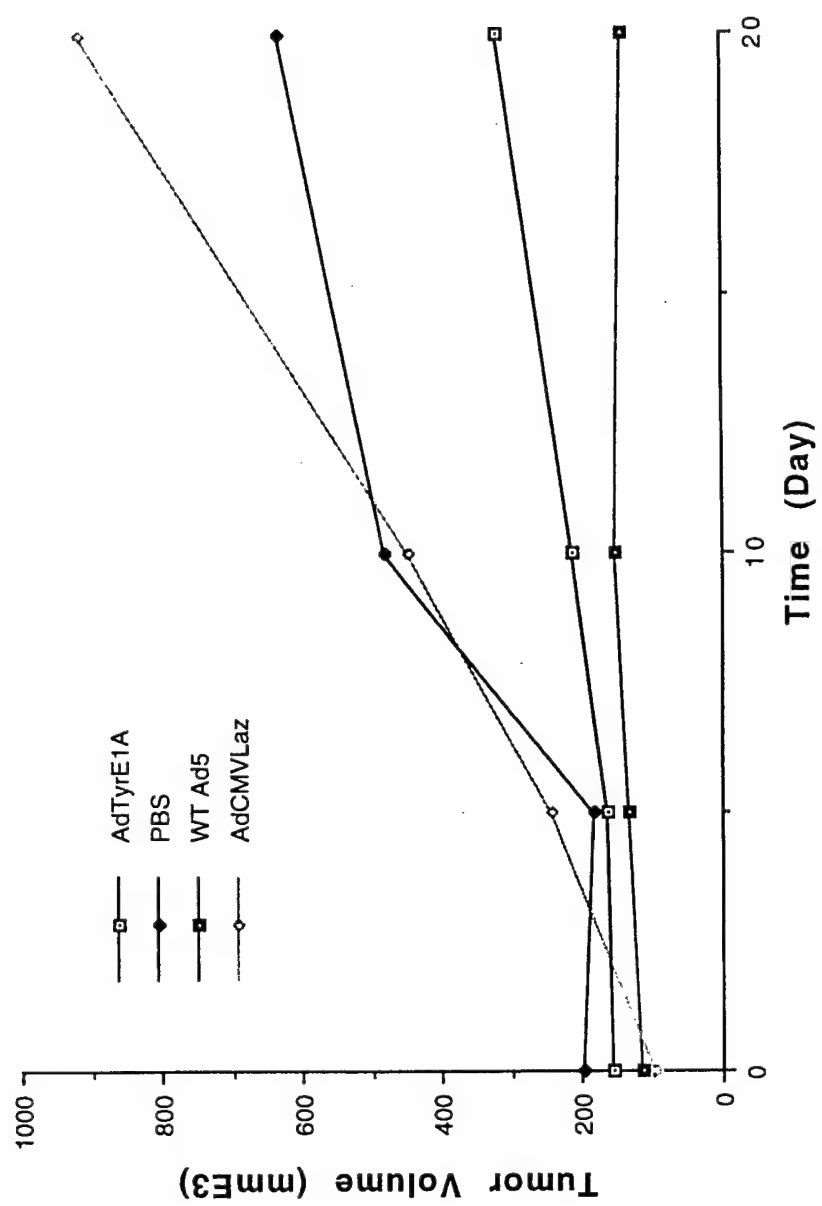


Figure 9

The Use of the L-Plastin Promoter for Adenoviral-mediated, Tumor-specific Gene Expression in Ovarian and Bladder Cancer Cell Lines¹

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ABSTRACT

A 2.4-kb truncated L-plastin promoter was inserted either 5' to the *LacZ* gene (Ad-Lp-LacZ) or 5' to the cytosine deaminase (*CD*) gene (Ad-Lp-CD) in a replication-incompetent adenoviral vector backbone. Infectivity and cytotoxicity experiments with the LacZ and CD vectors suggested that the L-plastin promoter-driven transcriptional units were expressed at much higher levels in explants of ovarian cancer cells from patients and in established ovarian or bladder cancer cell lines than they were in normal peritoneal mesothelial cells from surgical specimens, in organ cultures of normal ovarian cells, or in the established CCD minimal deviation fibroblast cell line. Control experiments showed that this difference was not attributable to the lack of infectivity of the normal peritoneal cells, the normal ovarian cells, or the minimal deviation CCD fibroblast cell line, because these cells showed expression of the *LacZ* reporter gene when exposed to the replication-incompetent adenoviral vector carrying the cytomegalovirus (CMV)-driven *LacZ* gene (Ad-CMV-LacZ). The Ovar-5 and Skov-3 ovarian cancer cell lines exposed to the Ad-Lp-CD adenoviral vector were much more sensitive to the prodrug 5-fluorocytosine (5FC), which is converted from the 5FC prodrug into the toxic chemical 5-fluorouracil, than was the CCD minimal deviation fibroblast cell line after exposure to the same vector. A mouse xenograft model was used to show that the Ad-Lp-CD vector/5FC system could prevent engraftment of ovarian cancer cells in nude mice. Finally, injection of the Ad-Lp-CD vector into s.c. tumor nodules generated a greater reduction of the size of the tumor nodules than did injection of the Ad-CMV-LacZ vectors into tumor nodules. The Ad-Lp-CD vectors were as suppressive to tumor growth as the Ad-CMV-CD vectors. These results suggest that an adenoviral vector carrying the *CD* gene controlled by the L-plastin promoter (Ad-Lp-CD) may be of potential value for the i.p. therapy of ovarian cancer.

INTRODUCTION

Adenoviral vectors are currently among the most frequently used vectors in the gene therapy of cancer because of their high titers, ease of production, high infection efficiency for epithelial neoplastic cells, and the fact that their transcriptional units can be expressed extra-chromosomally in nondividing cells. A possible disadvantage of this vector is that its broad host range also results in infection of both the intended tumor cells as well as of the surrounding normal tissues (1-3). This limits the utility of these vectors, especially when the vector gene products are designed to sensitize tumor cells to chemotherapy or to radiation therapy, because of the unwanted toxicity thereby generated in the normal cells.

One way to circumvent this limitation would be to use a tissue-specific transcriptional promoter active only in the target tumor cells. Our laboratory has constructed adenoviral vectors in which the L-plastin promoter is used to activate the expression of therapeutic transgenes in neoplastic but not in normal epithelial cells. L-plastin, which belongs to a family of genes which encode actin-binding proteins, was discovered by Leavitt (4) and his colleagues (5, 6). The only normal cell in which this protein is detectable is the mature leukocyte. This protein has been demonstrated to be present in >90% of epithelial neoplastic cells and is not found in normal epithelial cells. Therefore, the L-plastin promoter may be of potential utility in cancer gene therapy because it can be used to drive the expression of heterologous genes in a tumor-specific manner in the context of recombinant adenoviral vectors. Chung *et al.*, in our laboratory, had reported previously that the *LacZ* gene, when driven by the L-plastin promoter, is expressed in ovarian cancer cells, but not in normal mesothelial peritoneal cells, obtained at the time of surgical resection of ovarian cancer from patients (7).

We now are reporting the results of experiments based on replication-deficient adenoviral vectors that contain either a *LacZ* reporter gene or a *CD*³ therapeutic transcriptional unit regulated by a 2.4-kb fragment of the L-plastin promoter in bladder and ovarian cancer cell lines, in explants of normal and neoplastic ovarian primary tissue in organ culture, and in ovarian cancer established cell lines in a nude mouse-human tumor xenograft animal model. *CD* is a bacterial gene which converts 5FC, which is nontoxic to cell lines and primary cells, into 5FU, a compound which is toxic to most cells (2, 8). The levels of phosphorylated 5FU generated within *CD*-positive cells are sufficiently high that even nondividing cells die because of disruption of mRNA processing and protein synthesis.

The results of these experiments have shown that:

(a) the level of the L-plastin promoter driven the *LacZ* heterologous reporter gene expression is lower in an established minimal deviation fibroblast cell line (CCD) when compared with a collection of established epithelial tumor cell lines derived from ovarian cancer and bladder cancer;

(b) the L-plastin promoter activates the *LacZ* and *CD* transcriptional units to a higher level in ovarian cancer cells than in monolayer and organ explant cultures of normal ovarian tissue or of normal peritoneal tissue; and

(c) the cytotoxic effect of replication-incompetent adenoviral vectors carrying the *CD* transcriptional unit driven by the L-plastin promoter is greater to ovarian cancer cells exposed *in vitro* to 5FC than to explants of normal peritoneum. In addition, the suppressive effect of the L-plastin-driven *CD* vectors on the *in vivo* growth of ovarian cancer cell lines is equal to that of the CMV-driven *CD*

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³The abbreviations used are: *CD*, cytosine deaminase; 5FC, 5-fluorocytosine; 5FU, 5-fluorouracil; CMV, cytomegalovirus; NBSC, new born calf serum; CAR, coxsackie B/adenovirus receptor; pfu, plaque-forming units; MOI, multiplicity of infection; X-Gal, 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside; ONPG, *O*-nitrophenyl- β -D-galactopyranoside.

vectors. These results suggest that adenoviral vectors carrying the CD transcription unit driven by the L-plastin promoter may be of use in the i.p. treatment of metastatic ovarian cancer.

MATERIALS AND METHODS

Cells and Cell Culture

Human bladder carcinoma cell lines (J82 and EJ) were obtained from Dr. Richard Cote of the University of Southern California, Los Angeles, CA. The CCD minimal deviation human fibroblast cell line, the 293 transformed human kidney cell line, and the Skov-3 human ovarian cancer cell line were obtained from American Type Culture Collection. The Hey cystadenocarcinoma papillary ovarian cancer cell line was obtained from Eva Sapi of the Department of Therapeutic Radiology at Yale University (New Haven, CT). J82, EJ, Hey, and 293 cells were propagated in DMEM (Life Technologies, Inc.) supplemented with 10% heat-inactivated NBCS obtained from Hyclone Laboratories, Inc. (Logan, UT). The Ovar-5 human epithelial ovary carcinoma cell line was obtained from Dr. Thomas C. Hamilton of the Fox Chase Cancer Center, Philadelphia, PA. Ovar-5 cells were grown in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% heat-inactivated NBCS. The Skov-3 human ovarian adenocarcinoma cell line was propagated in McCoy5A medium supplemented with 10% heat-inactivated NBCS. All cell cultures were maintained in a 5% CO₂, humidified tissue culture incubator at 37°C.

Chemicals and Reagents

5-FC, 5FU, fluorescein di- β -D-galactopyranose, and X-Gal were purchased from Sigma Chemical Co. The β -Galactosidase Assay Kit was purchased from Stratagene Company. 6-³(H)5-fluorocytosine (4.1 Ci/mmol) and 6-³(H)5-fluorouracil were purchased from Noravak Biochemicals Inc. of Brea, CA. Monoclonal antibodies to α v β 3 (LM609) and α v β 5 (PIF6) integrins were purchased from Chemi-Con International. A monoclonal antibody to the CAR, which binds the adenoviral fibrillar protein, was obtained from Dr. R. W. Finberg of the Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA.

Construction of Replication-incompetent Recombinant Adenoviral Vectors

The Ad-CMV-CD vector, which contained the CD gene controlled by a CMV promoter (7) in place of the adenoviral *E1A* and *E1B* genes, was obtained from the laboratory of Dr. Ron Crystal of the Cornell Medical School, New York, NY (8, 9). A similar adenoviral vector (Ad-CMV-LacZ) was engineered in our laboratory in which a β -galactosidase transcriptional unit was inserted into the *E1A* and *E1B* regions of the adenoviral vector backbone (7). Injae Chung of our laboratory truncated the 5-kb L-plastin promoter to a 2.4-kb fragment, which extended from nucleotide -2265 of the 5' region of the L-plastin promoter to +18 bp from the transcription initiation site of the *L-plastin* gene (7). The number of infectious adenoviral particles, expressed as plaque-forming units (pfu) present in the viral stocks, was determined by limiting dilution assay of plaque formation in 293 cells exposed to various dilutions of the vector (10, 11).

Analysis of Cellular Receptors on Tumor Cells That Participate in Vector Uptake

Mouse monoclonal antibodies to the α v β 3 (LM609) integrin and the α v β 5 (PIF6) integrin and to the CAR receptors were used to detect the density of the human α v β 3, α v β 5, and CAR receptors on the test cells. The FACS Star Flow Cytometer (Becton Dickinson) in the Yale Cancer Center FACS Core Laboratory (New Haven, CT) was used to determine the percentage of cells positive for each receptor.

β -Galactosidase Activity Assay

X-Gal Staining. Cells were washed in PBS, trypsinized, and the viable cell number determined by trypan blue exclusion using a light microscope. Cells (3×10^5) for each cell line were infected with varying ratios of pfu/cell (MOI) of the vector in DMEM supplemented with 2% NBCS for 90 min. After this,

the cells were plated in six-well plates in complete medium in duplicate cultures. After 48 h of incubation at 37°C in a 5% CO₂, humidified tissue culture incubator, the cells were fixed with ice-cold 2% paraformaldehyde/0.2% glutaraldehyde for 10 min. The level of β -Gal-expression cells was then assessed by staining the cultures with X-Gal and potassium-ferricyanide/ferrocyanide solution essentially as described previously (12, 13). The average number of β -Gal-expressing (blue) cells/well was determined by counting five separate microscopic high-power fields.

β -Galactosidase Assay (ONPG). Cells (5×10^5) were infected at 20 MOI with Ad-Lp-LacZ or Ad-CMV-LacZ in 2% serum for 90 min. PBS was used to wash the cells, which were seeded in six-well plates with the fresh culture medium. The cells were then incubated for 48 h, after which the β -galactosidase assay was conducted (β -Galactosidase Assay Kit, Stratagene). Briefly, the cells were washed in PBS and lysed in 200 μ l lysis buffer and the cell debris removed by centrifugation for 5 min. The cell lysate was diluted 10 times, and 15 μ l of the cell lysate were pipetted into a 96-well microtiter dish, 145 μ l of buffer A- β -mercaptoethanol mixture was added to each well with subsequent incubation for 5 min at 37°C. Fifty μ l of ONPG were added to each well, and the dish was incubated at 37°C for 25 min; the mixtures turned bright yellow. The reaction was terminated by adding 90 μ l of stop solution and the microtiter dish was scanned in the microtiter dish reader set at 405 nm, and the absorbance (OD) was determined.

The Effect of 5-FU Released from CD Vector-infected Cells on Uninfected Cells

To quantify the effect of 5-FU released from infected cells on uninfected cells, different cell lines were infected at varying MOI (20 MOI, 80 MOI, and 160 MOI) using the Ad-CMV-CD or Ad-Lp-CD vectors. The infected cells and the noninfected cells were mixed in varying ratios to generate 0, 5, 10, 20, 30, 40, 50, 60, and 100% infected cells (14, 15). Cells were then seeded in duplicate in six-well tissue culture plates and incubated for 24 h with subsequent incubation with 500 μ M/liter 5FC for 5 days. The number of surviving cells was determined using trypan blue exclusion.

Comparison of the 5-FU Sensitivity (IC₅₀) of Ovarian Cancer and Bladder Cancer Cell Lines with CCD (Minimal Deviation Fibroblast Cell Lines)

The concentrations of 5FU used for the cytotoxicity test (IC₅₀) were 100, 50, 10, 1, and 0.5 μ M. After 96 h, the cells were removed with trypsin-EDTA and the cell number calculated using the Coulter Counter ZM (Hialeah, FL).

The Toxicity of Adenoviral Vectors

Cells (2×10^5) were infected with the Ad-CMV-LacZ, Ad-Lp-LacZ, Ad-CMV-CD, or Ad-Lp-CD vectors at MOI of 0, 5, 20, 40, 80, and 160 for 90 min and then seeded in six-well plates in duplicate. Twenty-four h later, 0.5 mM 5FC was added to each well, and then the cells were incubated for 5 days. Then the cells were trypsinized, and the surviving cells were counted using trypan blue exclusion (16). We arbitrarily assigned a 100% value to the cells incubated at 0 MOI and calculated the percentage of viable cells in the cultures to which vector had been added.

Vector Studies in Monolayer Explant Culture

Biopsy samples were cut into small pieces. These pieces were then digested with collagenase to disaggregate the tissue. To test the sensitivity of the patient samples to infection and 5FC sensitization with the Ad-CMV-CD and Ad-Lp-CD vectors, the cells were grown in T25 flasks to 90% confluence. Then the cells were washed in PBS and exposed to vector directly for 90 min in the flasks containing DMEM supplemented with 2% NBCS. Then the cells were incubated for 5 days at 500 μ M/liter 5FC concentration, and the cell viability was determined by light microscopic examination.

Vector Studies on Organ Culture of Ovarian Cancer and Normal Ovarian Tissue

Each specimen was cut into pieces of approximately 1–2 mm³ and immersed in 4 ml of DMEM:Ham's F12 medium, which was supplemented with 10% charcoal-stripped serum (17). Cultures were incubated at 37°C in six-well

plates on a shaking platform for 24–48 h, after which the tissues were exposed to the Ad-CMV-LacZ or Ad-LP-LacZ viral vectors for 90 min in serum-free medium. The tissues were washed with PBS. Then tissues were incubated for 48 h in fresh culture medium. The tissues were then frozen in OCT, and X-Gal staining was used to measure the Ad-CMV-LacZ and Ad-LP-LacZ expression on the section slides.

Studies of *in Vitro* Vector-infected Ovarian Cancer Cell Line in Nude Mice

Ovar-5 tumor cell lines were infected *in vitro* at 100 MOI with either the Ad-Lp-LacZ or Ad-Lp-CD adenoviral vectors for 60 min, washed with PBS, and then resuspended in PBS (4×10^7 cells/ml PBS). Ten female nude mice 6–8 weeks of age (25–28 grams in weight), which were purchased from Cox, Inc., Cambridge, MA, were injected i.p. with 40 million Ovar-5 ovarian carcinoma cells previously infected at 100 MOI with the Ad-Lp-LacZ vector. An additional 10 26–28-gram mice 6–8 weeks of age were injected i.p. with Ad-Lp-CD-infected cells. From the second day, all 20 of the mice were injected once a day with 5FC at 500 mg/kg i.p. for 10 days. Three weeks after tumor cell injection, the 10 Ad-Lp-LacZ-injected mice and 7 of the Ad-LP-CD-injected mice were killed and autopsied. At the 50th day, another three Ad-LP-CD-injected mice were killed and autopsied.

In other experiments, five female nude mice were injected i.p. with 40 million Skov-3 cells previously infected *in vitro* with the Ad-Lp-CD vector at 80 MOI. Another five mice were injected i.p. with Skov-3 cells previously infected *in vitro* at 80 MOI with the Ad-Lp-LacZ vectors. Then all of the 10 mice were injected i.p. with 500 mg/kg of 5FC daily for 10 days. Three weeks later, the mice were killed and autopsied (18, 19).

Studies of *in Vivo* Intratumoral Injection of Adenoviral Vectors

EJ cells (5×10^6) in PBS were injected s.c. in 25 nude mice. Three weeks later, the tumor size (width and length) was measured, then the tumor volume (mm^3) was calculated according to the formula: Tumor volume = length \times width²/2 (20, 21). Then, tumor nodules in eight mice were injected with 10^8 pfu of the Ad-CMV-CD virus. Tumor nodules in an additional eight mice were injected with 10^8 pfu of the Ad-Lp-CD virus, and tumor nodules in another nine mice were injected with 10^8 pfu of the Ad-CMV-LacZ virus. After this, 500 mg/kg of 5FC was injected into the peritoneal cavity each day, once a day, for 5 days. Two weeks later, we measured the tumor size again and compared the tumor growth before and after the treatment with viral particles and 5FC. Another 20 nude mice were injected s.c. with 5×10^6 Ovar-5 tumor cells. After this, the same vector injections and 5FC treatments were conducted as for the EJ tumor cell in the nude mice. Autopsy of the mice was carried out, and H&E-stained sections of the tumor and the adjacent tissues were examined to measure the toxicity of the vectors.

RESULTS

Study of Factors Affecting Percentage of β -Galactosidase-positive Cells after Exposure to the Ad-CMV-LacZ or Ad-Lp-LacZ Vectors. The infectivity of cell lines by adenoviral vectors has been reported to be dependent on the presence of the CAR, which mediates the binding of the vector to the target cell (22–24), the level and functional state of both the $\alpha\beta 3$ and $\alpha\beta 5$ integrin receptors, which are important for endocytosis of the vector, and the release of the vector from the endosome (25–27). Cell lines in which the $\alpha\beta 3$ receptors are low or functionally inactive may have low levels of expression of vector transgenes, because the amount of vector DNA reaching the nucleus, where it is transcribed into mRNA, will be reduced in $\alpha\beta$ -deficient cell lines because of sequestration in the endosome.

To study the effect of these receptors on the uptake of the adenoviral vector into cancer cell lines and the subsequent expression of its LacZ transgene in target cells, the Ovar-5, Hey, and Skov-3 ovarian cancer cell lines, the EJ, and J82 bladder cancer cell lines, and the CDD minimal deviation cancer cell line were exposed to the Ad-CMV-LacZ vector. Then these cell lines were studied for the percent-

age of cells that were positive for β -galactosidase. We chose a vector with the CMV promoter, because this promoter is known to be active in most, if not all, mammalian cells. Differences in β -galactosidase in these cell lines would therefore be attributable to differences in binding and endocytosis of the vector or release of the vector from the post-entry endosome. As shown in Table 1, the cells of all of the established ovarian and bladder cancer cell lines studied had a high percentage of cells positive for the CAR receptor (except for the Hey ovarian carcinoma cell line, in which none of the cells were detectable as positive for CAR). Among the established carcinoma cell lines in which a high percentage of cells were positive for CAR, all of the cell lines except for the Ovar-5 cell line had $>80\%$ of the cells positive for the $\alpha\beta 5$ receptor. The percentage of Ovar-5 cells positive for the $\alpha\beta 5$ integrin receptor was 57%. The percentage of cells positive for the $\alpha\beta 3$ integrin receptor was more variable among the cell lines. Only one-half of the Ovar-5 cells were positive for the either of the integrin receptors.

Not surprisingly, a high percentage of the cells of all of the established tumor cell lines studied, except for the Hey cell line, were detectable as positive for β -galactosidase after exposure to the Ad-CMV-LacZ vector (see Table 1). This suggests that cell lines in which a high percentage of cells are positive for both the CAR and the $\alpha\beta 5$ integrin receptors will be infectible by the adenoviral vectors and therefore will score positive for the protein product of a vector transgene if the transcriptional promoter driving the expression of the transgene is very strong, as is the case with the CMV promoter. Surprisingly, as shown in Table 1, although only 30% of the cells of the CCD cell line are positive for the CAR receptor and only 63% of the CCD cells were positive for the $\alpha\beta 3$ integrin receptors, up to 70% of the CCD cells are positive for β -galactosidase after exposure to the Ad-CMV-LacZ vector. Experiments carried out previously in our laboratory have shown that the CCD cell line is infectible by the Ad-CMV-LacZ vector (7). This suggests that there may be a CAR-independent mechanism of binding of the adenoviral vector to the CCD cells, and that the strength of a transcriptional promoter may overcome in part the limitation imposed on transgene expression by a lower level of the $\alpha\beta 3$ receptor.

Comparison of Lac-Z Gene Expression Levels in Cell Lines Infected with Either the Ad-CMV-LacZ or the Ad-Lp-LacZ Vectors. Another factor that may alter the percentage of cells scoring positive for transgene expression after exposure to an adenoviral vector is the level of activity of the transcriptional promoter regulating the vector transgenes in these different cell lines. Because it had been reported that the *L-plastin* gene was detectable in most tumor cell lines, but not in any normal cells of the body except for the mature leukocyte (4, 6), the same cell lines exposed to the Ad-CMV-LacZ vector were also exposed to an adenoviral vector in which the LacZ gene was regulated by the L-plastin promoter (Ad-Lp-LacZ).

Table 1 Characterization of percentage of cells positive for the CAR, $\alpha\beta 3$, and $\alpha\beta 5$ receptors as measured by FACS analysis and study of infectivity of cells by Ad-CMV-LacZ Vector at 20 MOI as measured by β -galactosidase assay (X-Gal)

$\alpha\beta 3$, $\alpha\beta 5$, and CAR receptor levels were measured by mouse monoclonal antibodies, and the FITC-conjugated antimouse antibody was used to stain the cells. Then, FACS analysis was used to detect the percentage of the receptor-positive cells ($n = 2$). For infectivity, cells were exposed to virus in serum-free medium for 90 min at 20 MOI and incubated for 48 h in culture medium. Then, cells were stained by X-Gal analysis ($n = 2$).

	$\alpha\beta 3$	$\alpha\beta 5$	CAR	β -Gal
EJ	83 \pm 8	82 \pm 5	95 \pm 8	95 \pm 8
J82	56 \pm 6	78 \pm 7	80 \pm 10	88 \pm 10
Skov-3	64 \pm 6	91 \pm 8	87 \pm 7	85 \pm 11
Ovar-5	48 \pm 7	57 \pm 5	88 \pm 10	65 \pm 8
Hey	81 \pm 5	96 \pm 10	0	10 \pm 4
CCD	63 \pm 8	93 \pm 4	29 \pm 5	70 \pm 9

Table 2 Comparison of β -galactosidase levels in cell line exposed to Ad-CMV-LacZ or Ad-Lp-LacZ (ONPG, OD)

Cells were exposed in serum-free conditions for 90 min at 20 MOI. After 48 h of incubation in culture medium, the level of the β -galactosidase (ONPG) in each cell line was measured by optical density, as outlined in "Materials and Methods" ($n = 2$).

	Ad-CMV-LacZ	Ad-Lp-LacZ	Ratio of CMV/Lp
EJ	1.1 \pm 0.2	0.9 \pm 0.1	1.2
J82	1.0 \pm 0.1	0.4 \pm 0.1	2.5
Skov-3	0.9 \pm 0.1	0.4 \pm 0.1	2.2
Ovar-5	0.9 \pm 0.1	0.4 \pm 0.1	2.2
CCD	0.9 \pm 0.1	0.1 \pm 0.01	9

To determine whether the L-plastin promoter was selectively more active in epithelial neoplastic (ovarian and bladder cancer) cell lines than in minimal deviation fibroblast cell line (CCD), we tested the *LacZ* gene expression levels in the Ovar-5, EJ, J82, Skov-3, and CCD cell lines after exposure to either the Ad-CMV-LacZ or the Ad-Lp-LacZ vectors. We then calculated the ratio of β -galactosidase levels in cells infected with the Ad-CMV-LacZ, divided by the β -galactosidase levels in cells infected by the Ad-Lp-LacZ vectors, as an index of the L-plastin promoter strength in established ovarian or bladder cancer cell lines as compared with the minimal deviation CCD fibroblast cell line.

As shown in Table 2, the amount of β -galactosidase in the EJ, J82, Skov-3, Ovar-5, and CCD cell lines after exposure to the Ad-Lp-LacZ vector was less than for the same cells exposed to the Ad-CMV-LacZ vector. This suggested that the Lp promoter was less strong in all of the cell lines than the CMV promoter, thereby decreasing the percentage of cells that scored positive for the vector transgene protein product under any given level of integrin or CAR receptor representation or function. A comparison of the amount of β -galactosidase in each cell line after exposure to the Ad-CMV-LacZ was divided by that for the Ad-Lp-LacZ vector. This ratio was 2 in all of the established cancer cell lines except for the CCD cell line, in which the ratio was 9. One possible explanation for this difference was that the CCD cell line supported the expression of the Lp promoter to a much lesser extent than the CMV promoter.

The relatively low *LacZ* gene expression in the CCD cell line exposed to the Ad-Lp-LacZ vector is not attributable to the low infectivity by the Ad-Lp-LacZ vector, because, as shown in Tables 1

and 2 and in a previous publication from our laboratory (7), >70% of the CCD cells were positive for β -galactosidase after exposure of these cells to the Ad-CMV-LacZ vector, indicating that the CCD cells are infectible by adenoviral vectors. The ratio of β -galactosidase levels in Ad-CMV-LacZ-infected cells divided by the β -galactosidase levels in Ad-Lp-LacZ-infected cells was much higher in CCD than in cell lines derived from bladder cancer and ovarian cancer. These data suggest that the L-plastin promoter is much more active in epithelial neoplastic cell lines than in the CCD minimal deviation fibroblast cell line.

Studies of the Effect of 5FU Released from Infected Cells on Noninfected Cells. To monitor the effect of 5FU released from infected cells on the noninfected cells, mixtures of Ad-CMV-CD or Ad-Lp-CD vector-infected and -noninfected cells were generated and then exposed to 5FC. The CD protein converts the nontoxic prodrug 5FC into the toxic chemical 5FU. Unphosphorylated 5FU can be released from cells infected with the CD vector and taken up by surrounding uninfected cells and can kill the uninfected cells. This is called the bystander effect. As shown in Fig. 1, when as few as 5% of the population of Ovar-5 cell lines or the CCD minimum deviation fibroblast cell line infected with Ad-CMV-CD (160 MOI) vectors were mixed with 95% of uninfected cells, the majority of the cells were killed when cells were exposed for 5 days to 5FC at a 500 μ M concentration (18% Ovar-5 and 29% CCD cells survived). This suggests that only a few of these cells need to be infected with the Ad-CMV-CD adenoviral vector to generate sufficient levels of 5FU *in vivo* in static cell culture to kill the vast majority of infected as well as uninfected tumor cells. The high percentage of cells killed at low infectivity *in vitro* is attributable partly to the fact that the medium was not changed, and therefore the cells were exposed continuously to a high level of 5FU, which continues to increase with time. In these conditions, the high levels of 5FU released from a few Ad-CMV-CD vector-infected cells could kill all of the uninfected cells.

When the cell lines were infected with the Ad-Lp-CD vector, incomplete cell death was seen even at the highest MOI tested with the CCD human minimal deviation cell line. In contrast, almost all of the cells were eradicated at the highest (160) MOI when similar experiments were carried out with the Ad-Lp-CD vector in the Ovar-5 cell line (see the data in Fig. 1, A and C, 160 MOI). The

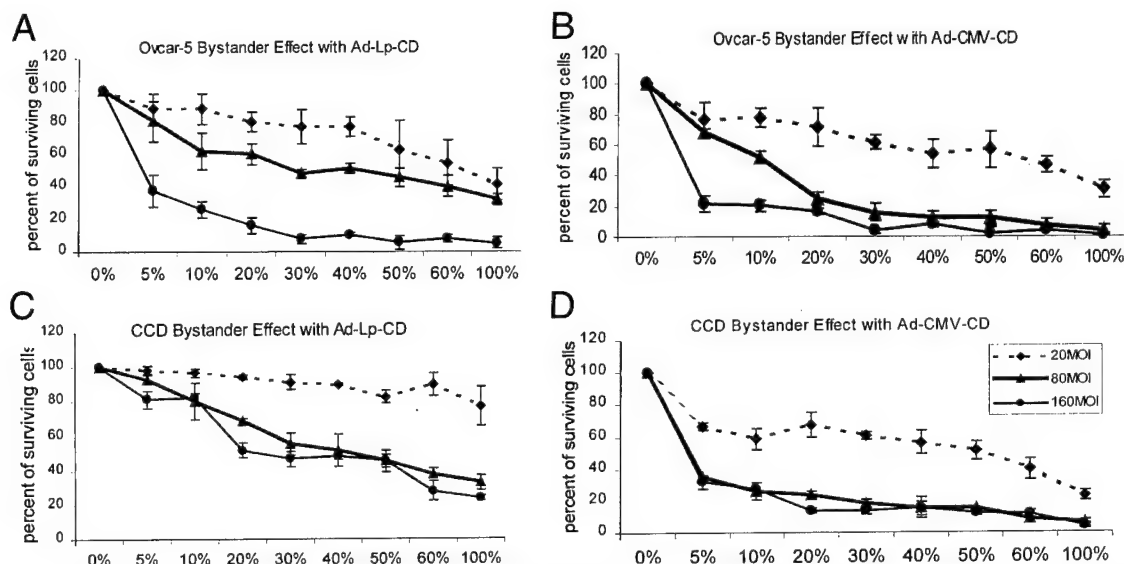


Fig. 1. Toxicity of vectors at varying levels of infected cells. Ovar-5 (A and B) or CCD (C and D) cell lines were infected at varying MOI (20, 80, and 160) using the Ad-CMV-CD (B and D) or Ad-Lp-CD (A and C) adenoviral vectors. The infected cells and noninfected cells were mixed in varying ratios to generate 0, 5, 10, 20, 30, 40, 50, 60, and 100% infected cells. Then cells were seeded in six-well plates and incubated for 5 days in 500 μ M 5FC. Then the cells were trypsinized, and surviving cells were counted by trypan blue exclusion.

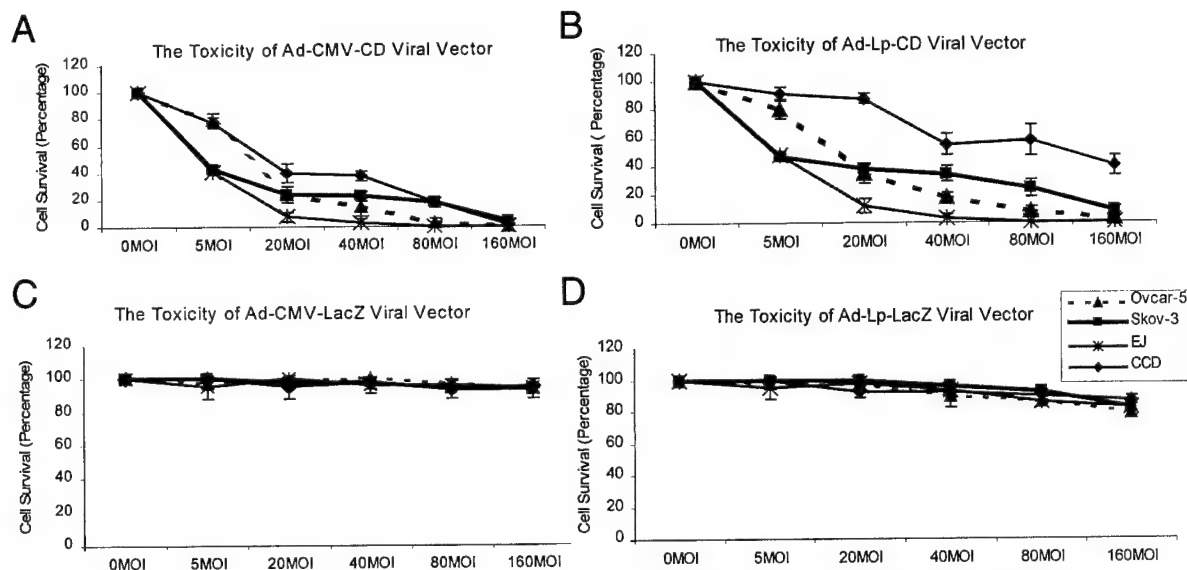


Fig. 2. Study of the toxicity of the control Lac-Z vector versus the CD vector. Cells (2×10^5) were infected at 0, 5, 20, 40, 80 and 160 MOI of vector with Ad-CMV-CD (A), Ad-Lp-CD (B), Ad-CMV-LacZ (C), Ad-Lp-LacZ (D) vectors for 90 min. Then cells were seeded in six-well plates in duplicate and incubated in 500 μ M 5FC for 5 days. The percentage of surviving cells was counted by trypan blue exclusion.

difference in the survival of cells between the CCD and Ovar-5 cells when exposed to the Ad-Lp-CD vector and 5FC was statistically significantly different at the $P < 0.001$ level. (An analysis of variance was used to determine whether the percentage of surviving cells was statistically significantly different when the Ad-CMV-CD or Ad-Lp-CD vectors were used to infect either the CCD or the Ovar cell lines.) This difference could be attributable to differences in the infectivity of the CCD cell line, the sensitivity of this cell line to 5FU, or a difference in the expression of the Lp-driven CD transcription units in the Ovar-5 or CCD cell lines. As shown in Fig. 1D, when the CCD cell line was exposed to 160 MOI of the Ad-CMV-CD vector and 5FC, complete killing of the CCD cells occurred at 100% infection. There were no differences between the cell kill in the Ovar-5 and CCD cell lines with the Ad-CMV-CD vector (compare the survival data at 160 MOI at 100% infection in Fig. 1, B and D). Therefore, the differences seen in A and C in the cell survival of the Ovar-5 and CCD cell lines after exposure to the Ad-Lp-CD vector are not attributable to differences between the CCD and Ovar-5 cell lines with respect to infectivity by the virus or sensitivity to 5FU, because complete killing is seen with the Ad-CMV-CD vector with the CCD cell line.

This suggests that the L-plastin promoter is less active in the CCD minimal deviation fibroblast cell line than in the established tumor cell line Ovar-5. The high levels of cell-killing at low infectivity that were seen *in vitro* with the Ad-Lp-CD vector will probably not be seen *in vivo* because of removal of the 5FU by blood flow and metabolic degradation.

5FU Sensitivity of Each Cell Line Expressed as IC_{50} . It is possible that the low-level cell death of the CCD cell line could be attributable to intrinsic resistance to 5FU toxicity, which is greater than that seen in the Ovar-5 or other established cancer cell lines. To test this, the intrinsic sensitivity of each cell line to 5FU was measured by seeding 3×10^5 cells in T25 flasks in triplicate, which were incubated for 96 h at different 5FU concentrations. The IC_{50} generated for 5FU in the J82 cell line is 55 μ M, for the EJ cell line is 30 μ M, for the Ovar-5 cell line is 3 μ M, for the Skov-3 cell line is 22 μ M, and for the CCD cell line is 15 μ M. The IC_{50} generated for 5FU in the CCD cell line (15 μ M) is less than that of several of the epithelial neoplastic cell lines (EJ, J82, and Skov-3), suggesting that the CCD cell line is as

sensitive to 5FU as the epithelial cancer cell lines. Thus, the low sensitivity of the CCD fibroblast cell line to the effect of the Ad-Lp-CD vector/5FC treatment is not attributable to a high level of resistance to 5FU, but rather to low levels of the protein product of the transcription units driven by the L-plastin promoter in the CCD cell line.

Study of 5FC Toxicity of the Adenoviral Vectors Carrying the CD Transcription Units. To test how much of the toxicity of the Ad-Lp-CD/5FC treatment was attributable to the toxicity of the vector backbone and how much was due to the protein produced by the CD transcription unit, the cell lines were infected with the Ad-CMV-CD, Ad-CMV-LacZ, Ad-Lp-CD, or Ad-Lp-LacZ vectors at different MOI. After this, the cell lines were incubated in medium supplemented with 500 μ M 5FC for 5 days. As shown in Fig. 2, no significant toxicity was seen with any of the cell lines when the backbone vector, Ad-CMV-LacZ, and Ad-Lp-LacZ were used (see Fig. 2, C and D). In contrast, when the cell lines were exposed to the Ad-Lp-CD or the Ad-CMV-CD vectors, nearly 100% killing of the cell lines after exposure to the vector and to 5FC was seen in all cell lines with both vectors, with the exception of the example of the CCD cell line after exposure to the Ad-Lp-CD vector and 5FC. The cell killing for CCD after exposure to the Ad-Lp-CD versus the Ad-CMV-CD vectors and 5FC (see Fig. 2, A and B) is statistically significantly different at the $P < 0.01$ level by the *t* test. No statistically significant differences were seen in any of the established tumor cell lines with respect to cell survival. This indicates that the toxicity seen in Fig. 2 after exposure to the Ad-Lp-CD or Ad-CMV-CD vectors is not attributable to the adenoviral backbone but to the action of the CD protein and 5FC. The $< 100\%$ cell kill in the example of the CCD after exposure to the Ad-Lp-CD vector and 5FC is most probably attributable to the lower level of transcriptional activation of the CD gene by the Lp versus the CMV promoter, as explained above. Thus, the toxicity seen in these experiments was not attributable to the viral backbone, but to the effect of the CD transcription units on the conversion of 5FC to 5FU. In addition, the 5FC toxicity generated by incubation of the Ad-Lp-CD transcription units in bladder cancer or ovarian cancer cell lines is statistically significantly higher than that seen in the CCD cell line.

Table 3 Percentage of cells in explant cultures of ovarian cancer cells and normal peritoneal cells which score positive for β -galactosidase after exposure to the Ad-CMV-LacZ and the Ad-Lp-LacZ vectors

Samples of primary tumor, metastatic tumor, and normal peritoneum were cut into small pieces. These pieces were then digested with collagenase to produce tissue disaggregation, and the resulting cells were cultured in RPMI 1640 with 10% NBCS. All experiments were performed at 90% confluence. Samples of ascites were divided into the T25 flasks directly and washed to remove debris after cell attachment. Cells were infected in the flasks for 90 min, and after 48 h of incubation, the positive cells were measured by X-Gal staining or FACS.

		Ascites	Primary tumor	Metastatic tumor	Normal peritoneum
Ad-CMV- β gal	X-Gal	50–80%	50–90%	45–85%	60–80%
	FACS	95%	94%	94%	
Ad-LP- β gal	X-Gal	10–35%	15–60%	15–45%	1–4%
	FACS	39%	83%	38%	
CMV/LP ratio	FACS	3/1	1/1	3/1	20–60/1

Percentage of Cells Detectable Positive for LacZ Expression in Primary Monolayer Cultures of Samples Obtained at Surgery from Normal Peritoneum and Metastatic Implants of Ovarian Cancer after Exposure to the Ad-Lp-LacZ or Ad-CMV-LacZ Vectors. Samples of metastatic tumor and normal peritoneum were collected from 16 ovarian cancer patients undergoing diagnostic or therapeutic laparotomy. The tumor was cut into small pieces and then digested with collagenase to disaggregate the tissue. The resulting cells were then cultured in RPMI 1640 supplemented with 10% NBCS. After culture, the cells were exposed at a MOI of 20 to the Ad-CMV-LacZ or Ad-Lp-LacZ vectors in T-flasks for 90 min. After 48 h of incubation, the percentage of β -galactosidase-positive cells was measured by X-Gal staining or FACS (28). A ratio of β -galactosidase-positive cells with the two vectors was generated by dividing the percentage of cells that were detectable as positive for β -galactosidase by FACS after exposure to the Ad-CMV-LacZ vector by the percentage of cells detectable as positive for β -galactosidase by FACS after exposure to the Ad-Lp-LacZ vector. As shown in Table 3, this ratio was at least 20–60-fold higher in the normal peritoneal cells than with any of the samples derived from ovarian cancer cells. These results indicate that the normal peritoneal cells are less able to support the expression of transgenes driven by the L-plastin promoter than are the ovarian cancer cells.

Table 4 Cytotoxicity in monolayer culture of normal peritoneum and ovarian cancer cells after expression to Ad-Lp-CD and Ad-CMV-CD vectors and 5FC (percentage of cells killed)

In Ad-CMV-CD- and Ad-Lp-CD-infected samples, 500 μ M 5-FC were added and incubated for 5 days, then the percentage of cells killed was estimated by comparing the percentage of cells which had died in the infected and uninfected control flasks.

	Ad-CMV-CD	Ad-Lp-CD
Ascites	98%	85%
Metastatic tumor	85%	70%
Primary tumor	90%	75%
Normal peritoneum	95%	10%

Cytotoxicity after Exposure of the Monolayer Cell Cultures of Normal Peritoneum and Ovarian Cancer from Surgical Specimens to the Ad-CMV-CD and Ad-Lp-CD Vectors. Samples of primary tumor, metastatic tumor, and normal peritoneum were collected from 16 ovarian cancer patients, and samples were prepared by the same methods as described previously. As shown in Table 4, when the cells were infected with the CD vectors and incubated for 5 days in the presence of 500 μ M 5FC in T25 flasks, the majority of the cells in the explant cultures of primary ovarian cancer, metastatic ovarian cancer, and ovarian cancer in ascites were killed by the Ad-CMV-CD or the Ad-Lp-CD vectors and 5FC. In contrast with the results in the ovarian cancer cells, in which the cell death with the Ad-CMV-CD and Ad-Lp-CD vectors was roughly the same, in the case of the biopsies of normal peritoneum, the cell death with the Ad-Lp-CD vector was only one-tenth of that seen with the Ad-CMV-CD vector. This indicates that the expression of the L-plastin promoter-driven CD gene is much lower in the peritoneum than in the ovarian cancer cells.

Studies of LacZ Vectors in Organ Cultures of Normal Ovary. Samples of ovarian cancer and normal ovary tissues were cut into small pieces then inoculated in organ culture for 24–48 h and infected with either the Ad-CMV-LacZ or the Ad-LP-LacZ vectors for 90 min. Then fresh medium was added, and the tissues were incubated for 48 h and then processed to the slide sections for study by the X-Gal staining reaction. The organ culture differs from the monolayer culture in that the organ culture is a three-dimensional array of cells. As shown in Fig. 3, there is a much stronger blue staining in the outer edges of the cell mass in the organ cultures of normal ovarian tissue with the Ad-CMV-LacZ vector, middle panel, than with the Ad-Lp-LacZ vector, right-hand panel. The results indicate that the CMV promoter is much more active in normal ovarian tissue than is the L-plastin promoter.

Killing Efficiency of Ovarian Cancer Tumor Cell Lines by 5FC/CD Vector System in Nude Mice. To test the efficacy of the Ad-Lp-CD replication incompetent vector system in a mouse human

Fig. 3. Ovarian organ cultures. Normal ovarian tissue was obtained from patients undergoing abdominal surgical procedures. The tissues were cut into small pieces and cultured in DMEM:Ham's F12 medium with 10% charcoal-stripped serum. Twenty-four to forty-eight h later, the tissues were infected with vectors for 90 min, washed with PBS, and then incubated for 48 h. Then the tissues were frozen in OCT and sectioned, after which the frozen sections were stained by the X-Gal reaction. Left, no vector; middle, Ad-CMV-LacZ vector; right, Ad-Lp-LacZ vector.

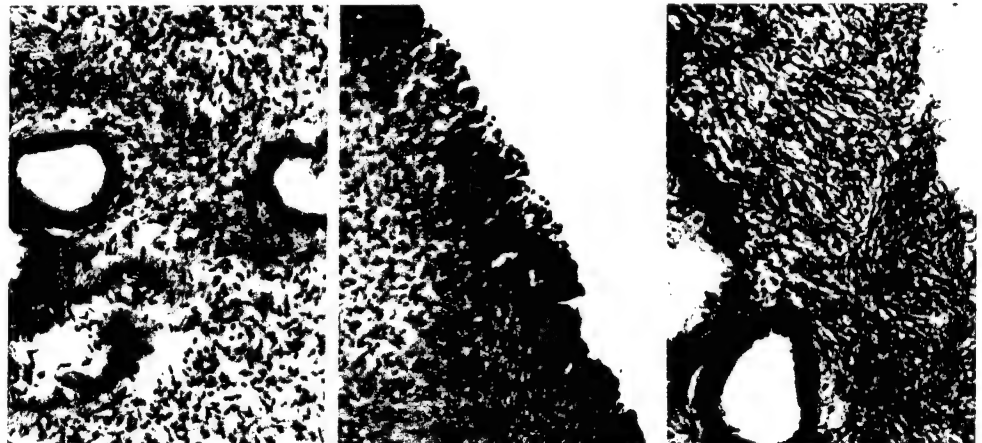


Table 5 Tumor growth in animals injected with adenoviral LP vectors (percentage of animals found to be positive for tumors)

The SCID mice were injected with 40 million Ovar-5 or Skov3 tumor cells, which had been infected previously *in vitro* with the Ad-Lp-LacZ vector or the Ad-Lp-CD vector. Starting on the second day, 500 mg/kg 5-FC was injected each day for 10 days. Animals were autopsied at 21 days after tumor cell injection, and the presence or absence of tumor nodules in the peritoneal cavity was assessed.

	Ad-Lp-LacZ-infected	Ad-Lp-CD-infected
Ovar-5 (100 MOI)	10/10 (100%)	0/10 (0%)
Skov3 (80 MOI)	5/5 (100%)	0/5 (0%)

tumor xenograft model, we first exposed the Skov-3 ovarian cancer cell line to the Ad-Lp-CD vector *in vitro* at 80 MOI or the Ovar-5 ovarian cancer cell line to the Ad-Lp-CD vector *in vitro* at 100 MOI by incubating the cells in the vector for 60 min. Then we injected 40 million of these *in vitro*-infected Ovar-5 vector infected cells into 10 nude mice or injected the *in vitro*-infected Skov-3 ovarian carcinoma cell line into 5 nude mice. One day after injecting the tumor cells, we initiated daily i.p. injections of 5FC into each of the animals to generate a daily peak of i.p. 5FC concentrations in the 500- μ M range. We carried on the daily i.p. 5FC injections for 10 days after the tumor injection. At 21 days after injection into the mice, we killed seven of the Ovar-5-injected mice and all five of the Skov3-injected mice and examined the peritoneal cavity for tumors. The remaining three Ovar-5 mice that were not killed at 21 days were killed at 50 days after tumor injection. As shown in Table 5, all of these animals were free of detectable tumor nodules, either at the gross morphological level or at the histopathological level.

In contrast, as shown in Table 5, all of the animals injected with the Ovar-5 *in vitro*-infected cell lines and all five of the animals injected with the Skov-3 *in vitro* tumor cells previously infected *in vitro* with

the control Ad-Lp-LacZ virus had detectable signs of tumor cell growth, either at the gross level or at the microscopic level. These data show that, in principal, it is possible to prevent engraftment of tumor cells in nude mice if all of the tumor cells are infected *in vitro* before i.p. injection of the cells with the replication-incompetent Ad-Lp-CD vector, and the animals are injected on a daily basis with the prodrug (5FC) which is converted into 5FU in the tumor cells.

To test the effect of administering the replication-incompetent Ad-Lp-CD and the Ad-CMV-CD vectors *in vivo* to preexisting s.c. nodules, we also tested the effect of intratumoral *in vivo* injection of established tumor nodules with the LacZ control vector, the CMV-CD vector, and Lp-CD adenoviral vector on the growth of the s.c. tumor nodules. As shown in Fig. 4, the tumors injected with the control Ad-CMV-LacZ vectors increased 3–4-fold after vector injection. In contrast, the size of both the Ovar-5 and the EJ cell tumor nodules injected with the Ad-CMV-CD or the Ad-Lp-CD vector was one-third to one-sixth of the size of the tumors injected with the Ad-CMV-LacZ vector. The growth of the Ovar-5 or EJ cancer cell lines after exposure to the Ad-CMV-LacZ vector was statistically significantly greater than the growth of the Ovar-5 or EJ cell lines after exposure to either the Ad-CMV-CD or the Ad-Lp-CD vectors, at the $P < 0.001$ level, by the t test of the ratios (two-tailed). There was no statistically significant difference in the growth of the Ovar-5 or the EJ cancer cell lines exposed to the Ad-CMV-CD *versus* the Ad-Lp-CD vectors.

To determine whether there was toxicity to the normal tissues, we studied histopathological sections of the tumor nodules and surrounding normal tissues by light microscopic examination after injection with the Ad-Lp-CD, Ad-CMV-CD, or Ad-CMV-LacZ vectors after exposure to 5FC. As seen in Fig. 5, *in vivo* injection of the Ad-Lp-CD

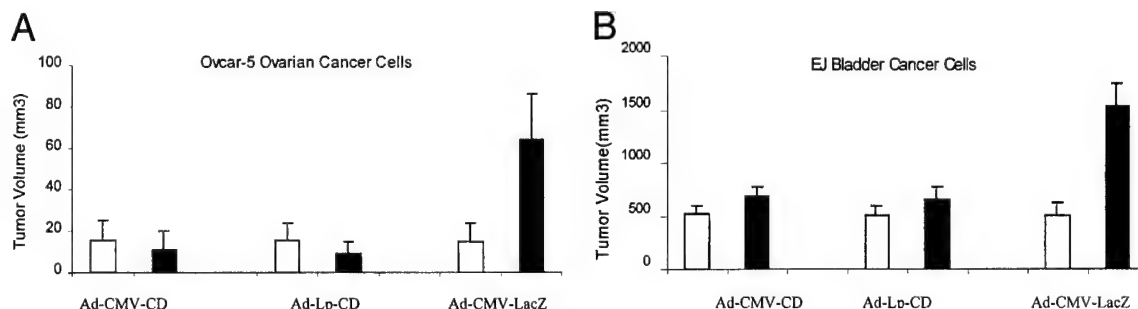
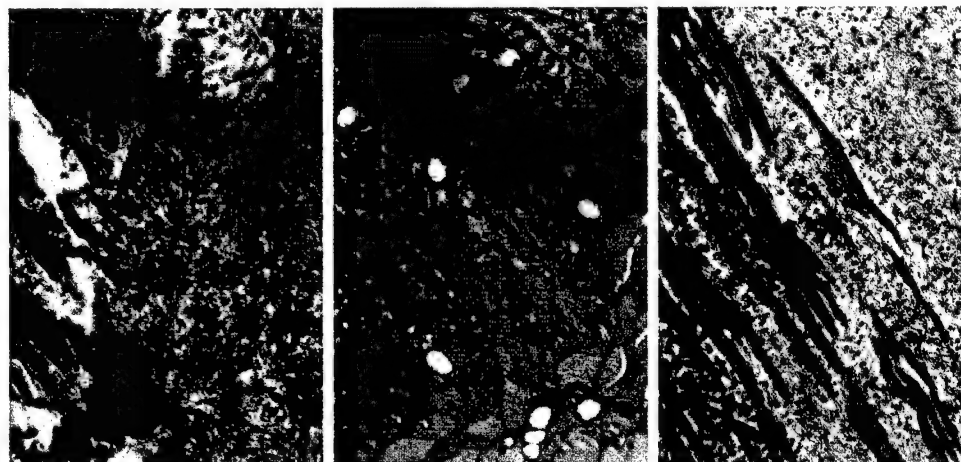


Fig. 4. Effect of *in vivo* injection of tumor nodules with adenoviral vectors. Ovar-5 (A) or EJ (B) cells (5×10^6) were injected s.c. into nude mice. After 3 weeks, the tumor nodules were measured. Then, 10^8 pfu of the Ad-CMV-CD, 10^8 pfu of the Ad-Lp-CD, or 10^8 pfu of the Ad-CMV-LacZ vectors were injected into each tumor nodule, and 500 mg/kg of 5FC was given i.p. once a day for 5 days. Seven days later, the tumor nodules were measured again. □ shows tumor volume before viral particles and 5FC treatment; ■ shows the tumor volume 7 days after exposure to viral particles and 5FC treatment.

Fig. 5. Vector toxicity to tumor cells and adjacent tissues. Ovar-5 cells (5×10^6) were injected s.c. After 3 weeks, 10^8 pfu of Ad-Lp-CD (right), 10^8 pfu of Ad-CMV-CD (left), or 10^8 pfu of Ad-CMV-LacZ (middle) vector were injected into each tumor nodule, and 500 mg/kg 5FC was given i.p. once a day for 5 days. Right (Ad-Lp-CD), most of the tumor cells are necrotic, whereas the adjacent muscle cells have a normal structure. Left (Ad-CMV-CD), after injection with the Ad-CMV-CD vector, the tumor cells are necrotic. Middle (Ad-CMV-LacZ), after injection of the Ad-CMV-LacZ vector, neither the muscle nor the tumor are necrotic.



vectors into the tumor nodules generated toxicity to the tumor cells (right). In Ad-CMV-CD *in vivo*-injected tumors, the tumor cells underwent necrosis (left). The toxicity to the tumor with the Ad-Lp-CD vectors was every bit as extensive in the tumor as that seen with the Ad-CMV-CD vector. This data shows that the toxic effect of the Ad-Lp-CD vector/5FC system is as great as that generated by the Ad-CMV-CD/5FC system, and the toxic effect of these two vectors is much greater than that seen with the Ad-CMV-LacZ vector.

DISCUSSION

A major limitation of the existing adenoviral vectors used for cancer gene therapy is the nonselective toxic action of these vectors. Attempts to render these vectors more selective for tumor cells and less toxic for normal cells has involved the use of tissue-specific transcriptional promoters to drive the therapeutic transcription units for these vectors. One of the limitations that have characterized these tissue-specific promoters is that the vectors carrying these tissue-specific therapeutic transcription units are usually less robust in their antitumor toxic action than nonselective viral transcriptional promoters.

We have reported the use of a tumor-specific rather than a tissue-specific transcriptional promoter for the regulation of an adenoviral therapeutic transcription unit. The L-plastin promoter was chosen because no normal tissue except for the mature leukocyte exhibits expression of the *L-plastin* gene. In contrast, most of the established cancer cell lines exhibit high levels of the expression of this gene. Experimental results published previously by our laboratory (7) have shown that a truncated L-plastin promoter retained its high activity within ovarian cancer cells, whereas it was relatively inactive in explants of normal peritoneal lining mesothelial cells. This data suggested that adenoviral vectors carrying therapeutic transcription units regulated by the L-plastin promoter might be useful in treating ovarian cancer.

When the L-plastin promoter is used to drive the expression of CD chemotherapy sensitization transcription unit in static cultures *in vitro*, only 50% of the cancer cells need to be infected to kill 100% of the epithelial neoplastic cells. In contrast, the percentage of cells that die in populations of CCD fibroblast cells is much lower, never reaching 100%. At all MOI tested, there are statistically significantly different levels of cell death generated by exposure to the Ad-CMV-CD *versus* the Ad-Lp-CD vectors and 5FC ($P < 0.001$), presumably because of the lower levels of activity of the L-plastin promoter in the CCD cell line (see Fig. 1 and 2). Control experiments have shown that the CCD cell line is as infectible by the Ad-CMV-LacZ as are the epithelial cancer cell lines (see Table 1). This indicates that low infectivity is not responsible for the low sensitivity of the CCD cell line to the Ad-Lp-CD vector. In addition, the intrinsic sensitivity of the CCD to 5FU toxicity directly added to the culture is not lower than that seen in the epithelial neoplastic cell lines. Thus, it appears that the level of expression of the L-plastin-driven genes in the CCD cell line is lower than that seen in the ovarian and bladder cancer cell lines and this is responsible for the differential effect of the Ad-Lp-CD and Ad-CMV-CD vectors in the CCD *versus* the epithelial neoplastic cell lines.

Studies in primary normal mesothelial cells and primary cell cultures of ovarian cancer cells show that the ratio of cytotoxicity with CMV-driven CD adenoviral vectors: to Lp-driven CD adenoviral vectors is highest in normal peritoneum (ratio of 20–60) as compared with 3–5 times in ovarian cancer cells in malignant ascites or in primary or metastatic ovarian cancer. The use of the Ad-Lp-CD vector to infect ovarian cancer cell lines *in vitro* before their injection into the i.p. cavity of 5FC-injected mice results in a suppression of the

engraftment of these ovarian cancer cells, whereas no sign of suppression of tumor growth occurred when the ovarian cancer cell lines were infected with the Ad-Lp-LacZ or Ad-CMV-LacZ control vectors.

These data suggest that, in principle, the L-plastin-regulated CD transcription units may selectively sensitize ovarian cancer cell lines to the effects of 5FC without significantly sensitizing the normal peritoneal surface cells to the effects of this 5FC/Vector system.

Many obstacles that remain to be overcome are pointed up by this data. The first is that, for such vectors to work *in vivo* in patients, some method must be developed for conferring conditional replication competency on these Lp-CD vectors so that they may infect 100% of the tumor cells when administered to patients with existing tumor *in vivo*.

The data in Fig. 3 shows that the expression of the reporter gene is seen only on the surface of an organ culture of cells infected with a replication-incompetent vector. Therefore, our laboratory is studying, on a preclinical level, several different types of adenoviral vectors that exhibit replication competency that is selective for the regulatory environment of the tumor cell. Our design is to use the L-plastin promoter to drive the expression of the adenoviral *E1A* gene, which is necessary for viral replication, as well as the CD chemotherapy sensitization gene. Such vectors may be useful in the i.p. therapy of ovarian cancer.

REFERENCES

- Descamps, V., Duffour, M. T., Mathieu, M. C., Ferabde, N., Cordier, L., Abina, M. A., Kremer, E., Perricaudet, M., and Haddada, H. Strategies for cancer gene therapy using adenoviral vectors. *J. Mol. Med.*, 74: 183–189, 1996.
- Koyama, F., Sawada, H., Hirao, T., Fujii, H., Hamada, H., and Nakano, H. Combined suicide gene therapy for human colon cancer cells using adenovirus-mediated transfer of *Escherichia coli* cytosine deaminase gene and *Escherichia coli* uracil phosphoribosyltransferase gene with 5-fluorocytosine. *Cancer Gene Ther.*, 7: 1015–1022, 2000.
- Xiang, J., Chen, Y., and Moyana, T. Combination immunotherapy for established tumors with engineered tumor vaccines and adenovirus-mediated gene transfer. *Cancer Gene Ther.*, 7: 1023–1033, 2000.
- Leavitt, J. Discovery and characterization of two novel human cancer-related proteins using two-dimensional gel electrophoresis. *Electrophoresis*, 15: 345–357, 1994.
- Lin, C. S., Chang, C. H., and Huynh, T. The murine L-plastin gene promoter: identification and comparison with the human L-plastin gene promoter. *DNA Cell Biol.*, 17: 9–16, 1997.
- Park, T., Chen, Z. P., and Leavitt, J. Activation of the leukocyte plastin gene occurs in most human cancer cells. *Cancer Res.*, 54: 1775–1781, 1994.
- Chung, I., Peter, E. S., Crystal, R. G., Pizzorno, G., and Deisseroth, A. Use of L-plastin promoter to develop an adenoviral system that confers transgene expression in ovarian cancer cells but not in normal mesothelial cell. *Cancer Gene Ther.*, 6: 99–106, 1999.
- Hirschowitz, E. A., Ohwada, A., Pascal, W. R., Russi, T. J., and Crystal, R. G. *In vivo* adenovirus-mediated gene transfer of the *Escherichia coli* cytosine deaminase gene to human colon carcinoma-derived tumors induces chemosensitivity to 5-fluorocytosine. *Hum. Gene Ther.*, 6: 1055–1063, 1995.
- Tong, X., Engehausen, D. G., Freund, C. T., Agoulnik, I., Guo, Z., Oehler, M. K., Kim, T. E., Hasenburger, A., and Kieback, D. G. The efficacy of adenovirus-mediated gene therapy of ovarian cancer is enhanced by using the cytomegalovirus promoter. *Anticancer Res.*, 18: 719–725, 1998.
- Graham, F. L., and Prevec, L. Manipulation of adenovirus vectors. In: E. J. Murray (ed.), *Methods in Molecular Biology*, Vol. 7, pp. 109–128. Clifton, NJ: The Human Press Inc., 1991.
- Neering, S. J., Hardy, S. F., Minamoto, D., Spratt, S. K., and Jordan, C. T. Transduction of primitive human hematopoietic cells with recombinant adenovirus vectors. *Blood*, 88: 1147–1155, 1996.
- Couffignal, T., Keaney, M., Sullivan, A., Silver, M., and Isner, J. M. Histochemical staining following LacZ gene transfer underestimates transfection efficiency. *Hum. Gene Ther.*, 8: 929–934, 1997.
- Theodore, L., Rehmetulla, A., Emily, Y., Trosko, J. E., and Stetson, P. L. Preferential cytotoxicity of cells transduced with cytosine deaminase compared to bystander cells after treatment with 5-fluorocytosine. *Cancer Res.*, 58: 2588–2593, 1998.
- Kianmanesh, A. R., Perrin, H., Panis, Y., Houssin, D., and Klatzmann, D. A. "Distant" bystander effect of suicide gene therapy: regression of nontransduced tumors together with a distant transduced tumor. *Hum. Gene Ther.*, 8: 1807–1814, 1997.
- Ramesh, R., Marrogi, A. J., Munshi, A., and Freeman, S. M. Potentiation of the bystander effect by immunization combined with suicide gene therapy. *Adv. Exp. Med. Biol.*, 451: 125–131, 1998.
- Parsels, L. A., Kowalski, M. P., Wagner, L. M., Parsels, J. D., Maley, F., and Maybaum, J. *Escherichia coli* thymidylate synthase expression protects human cells

- from the cytotoxic effects of 5-fluorodeoxyuridine more effectively than human thymidylate synthase overexpression. *Cancer Gene Ther.*, 7: 1179–1187, 2000.
17. Sapi, E., Flick, M. B., and Rodov, S. Expression of CSF-1 and CSF-1 receptor by normal lactating mammary epithelial cells. *J. Soc. Gynecol. Investig.*, 5: 94–101, 1998.
 18. Kelly, L., Molpus, D. K., Leonard, A., and Hasan, T. Characterization of a xenograft model of human ovarian carcinoma which produces intraperitoneal carcinomatosis and metastases in mice. *Int. J. Cancer*, 67: 588–595, 1996.
 19. Hwang, E. S., Kim, J. S., You, E. H., Lee, S. H., and Lee, J. H. Intraperitoneal gene therapy with adenoviral-mediated *p53* tumor suppressor gene for ovarian cancer model in nude mouse. *Cancer Gene Ther.*, 6: 172–178, 1999.
 20. Pang, S. Targeting and eradicating cancer cells by a prostate-specific vector carrying the diphtheria toxin A gene. *Cancer Gene Ther.*, 7: 991–996, 2000.
 21. Martin, D. S., Stolfi, R. L., Sawyer, R. C., and Yang, C. W. Therapeutic utility of utilizing low doses of *N*-(phosphonacetyl)-L-aspartic acid in combination with 5-fluorouracil: a murine study with clinical relevance. *Cancer Res.*, 43: 2317–2321, 1983.
 22. Bergelson, J. B., Krithivas, A., Celi, L., Wickham, T., Crowell, R. L., and Finger, R. W. The murine CAR homolog is a receptor for coxsackie B viruses and adenoviruses. *J. Virol.*, 1: 415–419, 1998.
 23. Bergelson, J. M., Cunningham, J. A., Horwitz, M. S., Crowell, R. L., and Finger, R. W. Isolation of a common receptor for coxsackie B viruses and adenoviruses 2 and 5. *Science* (Wash. DC), 275: 1320–1323, 1997.
 24. Roelvink, P. W., Lizonova, A., Lee, J. M., Li, Y., Kovesdi, I., and Wickham, T. J. The coxsackievirus-adenovirus receptor protein can function as a cellular attachment protein for adenovirus serotypes from subgroups A, C, D, E, and F. *J. Virol.*, 10: 7909–7915, 1998.
 25. Garcia-Sanchez, F., Fu, S. Q., Nanakorn, T., Leffert, J. J., Cote, R., Crystal, R., and Deisseroth, A. Cytosine deaminase adenoviral vector and 5-fluorocytosin selectively reduce breast cancer cells 1 million-fold when they contaminate hematopoietic cells: a potential purging method for autologous transplantation. *Blood*, 92: 672–682, 1998.
 26. Wickham, T. J., Mathias, P., Cheres, D. A., and Nemerow, G. R. Integrins avb3 and avb5 promoter adenovirus internalization but not virus attachment. *Cell*, 73: 309–319, 1994.
 27. Wickham, T. J., Filardo, E. J., Cheres, D. A., and Nemerow, G. R. Integrin avb5 selectively promotes adenovirus mediated cell membrane permeabilization. *J. Cell Biol.*, 127: 257–264, 1993.
 28. Roederer, M., Fiering, S., and Herzenberg, L. A. FACS-gal: flow cytometric analysis and sorting of cells expressing reporter gene constructs. *Methods*, 2: 248–260, 1991.

Molecular chemotherapy

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ABSTRACT For the past 50 years, during which chemotherapy has been administered for the treatment of cancer, the paradigm driving the selection of chemotherapeutic agents was their capability to damage the DNA of dividing cells. In addition, chemotherapeutic agents were studied for the selectivity of their toxic effect to the cancer cell versus the normal cell. Because many of the normal cells in the body shared the phenotype of cellular division with the tumor cells, the drugs developed for the treatment of cancer displayed only relative selectivity for the cancer cell. The advent of structural biology and computation chemistry to drug development, and the explosion of information about the molecular and genetic changes acquired in the cancer cell, ~~has~~ now produced the opportunity to design drugs for cancer treatment which ~~are designed~~ specifically to block the effects of the signals within cancer cells which drive the evolution of the disease process to unregulated cell growth. The implementation of this new mode of drug design to the field of cancer chemotherapy is the subject of this chapter.

Key words molecular chemotherapy, gynecologic malignancies

INTRODUCTION One of the ~~greatest~~ obstacles of the success of chemotherapy for cancer treatment is that resistance evolves very rapidly following exposure to chemotherapy, or is present at the start of chemotherapy. Thus, remissions are follo-

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are followed

by recurrences and the phenotype of the recurrent disease is much more resistant than that of the original tumor population.

assumed to be due

This pattern of recurrence is often interpreted as indicating that the genetic instability of tumor cells is responsible for the evolution of resistance in epithelial neoplasms to the effects of chemotherapy. This formulation holds that the imposition of negative selection on any population of genetically unstable cells leads to the emergence of low frequency clones of tumor cells which may have acquired higher levels of resistance to the negative selective pressure than the cells which are dominant in the population at the initial time of exposure to that negative selective pressure.

NORMAL TISSUES ASSOCIATED WITH TUMORS ARE NOW TARGETS FOR MOLECULAR CANCER TREATMENT

The acquisition of additional somatic mutations due to the error prone nature of DNA replication, the increased proliferative activity of tumor populations, and the diminished repair capacity of mutant populations of neoplastic cells, will lead to endless patterns of increasing resistance in tumor cells to the effects of chemotherapy. This has led many therapists to consider changing the target of therapy from the tumor cells themselves to alternative tumor associated populations or normal cells upon which the continued proliferation or survival of the tumor population depends. One of these targets is the vasculature associated with tumor tissue. Another normal target which can affect the natural history of a tumor population is the systemic immune response.

DEVELOPMENT OF MOLECULAR THERAPY INVOLVING TUMOR VESSELS Folkman (1) has emphasized the important role that the elaboration of the tumor associated vasculature plays in the development of vasculature on the growth of solid tumors. It is clear that for tumor cells to grow above a minimal size, which is determined by the diffusion limits of nutrients and oxygen, the tumor must stimulate the development of vasculature as the tumor grows. The normal endothelial cells start to extend into the growing tumor mass, perhaps preceded by the influx of pericytes. Folkman (1) has focused on the development of agents which are known to suppress the growth of endothelial cells, the so called tumor neovasculature. Although this "anti-angiogenic" therapy has displayed activity in animal models, it has the theoretical limitation that anti-angiogenic therapy would only result in stabilization of the size of tumor nodules. This is due to the fact that the anti-angiogenic therapy does not affect the existing vasculature, but only the development of additional vasculature which allows the tumor to grow.

This recognition has led to the study of the tumor vasculature, both in the growing zone of angiogenesis and in the stable

non-proliferating zone of the established tumor mass. Interestingly, both the established vasculature of tumor tissue, as well as the growing zone of tumor neovasculature, display proteins on the endothelial surface that do not appear on the surface of endothelial cells which are part of vessels of normal tissue. *Schnitzer and co-workers* (2) have reported the appearance of a number of novel proteins on the surface of tumor cell vasculature. Other workers have developed data suggesting that the established vessels of tumor tissues display tissue factor, a transmembrane protein not usually exposed to the luminal surface of normal vessels in the absence of vessel injury or inflammation (3-4). When tissue factor is exposed in the setting of vascular injury or inflammation to the luminal surface of vessels, it binds to factor VII, which is a factor of intravascular coagulation. Upon binding of factor VII to tissue factor, factor VII is activated ~~and~~ thereby ~~triggers~~ ^{Triggers} the ~~so-called~~ extrinsic pathway of coagulation which results in the conversion of fibrinogen into a fibrin clot.

The exposure of the blood stream to tissue factor and the consequent binding and activation of factor VII also has an effect on the activation of platelets as well. Since tissue factor does not appear on the endothelial cells of vessels associated with normal tissue, the presence of tissue factor on the luminal surface of the endothelium of tumor associated vessels has provided a target to which to selectively deliver anti-cancer therapeutic reagents ~~to tumor tissue~~.

Another way of identifying molecular targets which can be used to deliver therapeutics to tumor vasculature is in vivo phage display. *Pasqualini and Ruoslahti* (5) have injected into tumor bearing mice a library of bacteriophage, which display ~~library of~~ random peptides on the surface of the coat proteins of the bacteriophage. Injection of these bacteriophage showed ~~in their initial publication~~ a four-fold difference in the binding of the various bacteriophage clones to vessels of different tissues. Also, peptides which bind to known proteins present on tissues (6) were isolated (e.g. the RGD peptide which is known to bind to the alpha V beta 3 integrin receptor present on the endothelial cells of some tissues). These workers are attempting to determine if this pattern of binding of random peptides to the endothelial cells of different types of tissues can be used to target therapeutics to tumor associated vasculature.

Other Tumor Vascular Targeting

Approaches which may reach the clinic include adenoviral vectors which are engineered to bind to tumor neovasculature, or which carry transcription units encoding proteins directed to the tumor vasculature, or chemicals ~~or proteins~~ which can target directly the tumor vasculature or angiogenesis.

At the beginning of the field of anti-angiogenesis therapy, studies focused on natural products and low molecular weight chemicals which target tumor vessels or angiogenesis recep-

tors. *Folkman* (1) has been responsible for launching therapeutic trials of number anti-angiogenic agents including endostatin and angiostatin ~~proteins~~ which are derived from normal proteins in human beings. The animal experiments designed to analyze the efficacy of these proteins involved the infusion of the recombinant proteins on a ~~twice a day~~ ^{daily} basis to patients with a wide spectrum of malignancies. The data so far from these clinical phase I trials suggest that the incidence of ~~complete or~~ partial responses will be low, and the most that one can hope for may be stabilization of disease. ~~That~~

These results have prompted investigators to combine anti-angiogenic proteins with chemotherapy or radiation therapy. Such trials are currently underway. The second direction is to engineer an angiostatin or endostatin expression unit in the backbone of the adenoviral vector (7). These efforts are currently at the preclinical and animal model stage. Presumably the advantage of using vectors for the delivery of the recombinant endostatin or angiostatin proteins is that instead of a ~~two~~ ^{Tc} daily infusions, which results in wide fluctuations of the intravascular levels of these proteins, the use of a vector produces continuous levels of the therapeutic protein. The limitation of this approach may be the absolute level of the protein can be maintained and the time over which one can administer these ~~pro~~ ^{ThqT} vectors. In addition, ~~because~~ ^{at} the adenovirus stimulates a strong immune response, there may be a limit to the number of injections one can administer ~~of these vectors~~ before the host immune response may block the effect of the vectors.

~~Therefore~~ ^{a1} A final vector approach to tumor neovasculature is to engineer adenoviral vectors carrying transcription units encoding chimeric immunoconjugate molecules which are composed of an aminoterminal targeting domain and a carboxylterminal function domain. In one of these, *Hu et al.* (8-9) have introduced into a replication incompetent adenoviral vector a transcription unit which encodes the aminoterminal domain of a modified factor VII carrying a loss of function mutation, which can bind to the tissue factor on the surface of the tumor associated vasculature, linked to the carboxylterminal end of the IgG Fc fragment of an immunoglobulin molecule. The strategy is to inject the adenoviral vector encoding this immunoconjugate molecule into a subcutaneous tumor nodule. The immunoconjugate transcription unit is engineered such that the fusion immunoconjugate factor VII/Ig G Fc protein is secreted from the vector infected tumor cells into the systemic circulation where it is free to bind to vessels associated with tumor cells all over the body. Preclinical animal studies of this approach so far published (8-9) show that metastatic deposits far from the nodules undergo regression. This approach is undergoing extensive preclinical testing at this time.

A non-vector approach is to design low molecular weight chemicals which can target the endothelial surfaces of vessels

associated with tumor cells. These drugs are based on the analysis of the peptides which are found to bind to tumor associated vessels, or the computational analysis of receptors which are participated in angiogenesis. These directions will be pursued in the next two to three years.

DEVELOPMENT OF MOLECULAR THERAPY INVOLVING THE IMMUNE RESPONSE The other normal tissue which can affect the natural history of tumor cells is the immune response. Many workers are exploring ways of producing novel drugs, vectors and recombinant proteins which can be used to target the immune response against the tumor cells.

The explosion of information about antigen presentation in normal tissues and tumor tissues suggests that there are many abnormalities in the antigen presentation of tumor antigens. Decreases in the level of surface cytoadhesion molecules necessary for presentation of antigens are characteristic of tumor cells as compared to normal cells. Another major problem is that the tumor antigens are transmitted only very inefficiently to the intracellular environment of professional antigen presentation cells, the dendritic cells. The start of the immune response is the presentation of the target antigen by specialized cells which display the mechanisms, which can result in the display of antigenic peptides in a context which can be recognized by clones of T cells competent to respond to these antigens (10).

This system works very well to limit and control viral infections. Following the infection of respiratory mucosal cells by viruses, the dendritic cells, which are present in large numbers in these surfaces, are infected during the initial stages of a viral infection. The replication of the vectors then results in the elaboration of high levels of viral proteins within the dendritic cells. This viral infection of dendritic cells results in the appearance of high levels of viral specific peptide antigens bound to histocompatibility presentation molecules associated with beta 2 microglobulin. This generates a systemic immune response against the virus and virally infected cells.

Although dying tumor cells can release tumor associated proteins which can be inefficiently taken up by antigen presenting cells, this process is very inefficient and therefore the levels of these tumor associated proteins within the antigen presenting cells is very low. Thus, there is no mechanism through which tumor associated proteins can reach levels within the antigen presenting cells which are in excess of the endogenous proteins of the antigen presenting cell. This results in levels of tumor-associated peptides on the bound to antigen presentation molecules which are low in comparison to non tumor associated proteins on the surface of antigen presenting cells. The only circumstance under which tumor cell antigens can be

presented at very high levels on antigen presenting cells is in the neoplastic diseases in which the dendritic cells can differentiate from the neoplastic population itself (11). In the case of lymphomas and leukemias, in which this can take place, there is good evidence that methods that depend on the activation of the dendritic cells, or involve the infusion of T cells exposed to such dendritic cells, can induce regressions of established tumors, or prevent tumors from engrafting of tumor cells in animal models (12).

For most tumors however, the only mechanism which can deliver exogenous tumor associated proteins to the intracellular compartment of the dendritic cell such that they can appear on the surface of the dendritic cell in a way that triggers a T cell cellular immune response to the tumor antigens, is the process which is called cross priming (13).

Purified or recombinant proteins, peptides and gangliosides have been used in an attempt to activate the cellular immune response. These types of peptides administered alone or with adjuvants, or peptides or proteins linked to proteins which are linked to molecules which can target tumor associated antigens sites on antigen presenting cells, or to subcellular locations within dendritic cells, which optimize the presentation of tumor associated antigens to cytotoxic T cell precursors are the subject of intense preclinical and clinical investigation at this time (14-15).

It is also logical that many papers have appeared which report animal model data based on the use of adenoviral vectors carrying transcription units which code for tumor associated antigens. These adenoviral vectors are then used to infect the dendritic cells of the tumor host, and these infected dendritic cells are infused into the intravenous space, injected into the lymph nodes, into tumor cells, or injected into the subcutaneous or intradermal space. Dendritic cells have also been loaded by fusing the dendritic cells with tumor cells to introduce the tumor antigens to the intracellular space of the antigen presentation cell (16). Finally, incubation of the antigen presenting cell with recombinant peptides that are known to bind to the HLA membrane protein peptide binding pocket has been used to load dendritic cells with tumor associated antigens. These immunological molecular targeting approaches to the therapy of the cancer cell are currently under study in preclinical and clinical trials.

APPROACHES FOR THE DESIGN OF MOLECULAR TARGETED DRUG THERAPY Computational and combinatorial chemistry can be used for the development of molecular targeting approaches to the tumor cell. The approach differs in cases in which there is structural data available about the target protein upon which the design of drugs can be based, and in those cases in which there is no data about the structure of the target.

DESIGN OF MOLECULAR THERAPEUTICS STARTING FROM PROTEIN STRUCTURE DATA. Ample data now exists that supports the contention that oncoprotein structures can be used to design inhibitors of enzymes or proteins associated with human disease. The first example is the use of X-ray crystal structure to design inhibitors of proteases which are necessary for the continued replication of the human immunodeficiency virus. Recently, *Thiesing et al.* (17) has brought forward a drug, STI 571, which blocks the binding of ATP to the ATP binding site of the enzyme pocket of the p210bcrabl kinase.

Treatment of CML.

Models for the development of drugs which can bind to targets which are unique to tumor cells are under development for many different types of targets in tumor cells. This model is being used for growth factor receptors, metastasis receptors, purine pockets in membrane G protein and oncoprotein tyrosine specific protein kinases. Pockets or receptors are the best targets for this type of design rather than the protein contact points which often do not have single points at which the stability of a complex can be affected.

In addition to the use of computational analysis of purine pockets to design chemicals which can inhibit the function of oncoproteins, chemical combinatorial libraries can be generated which are biased on the basis of the structure of lead compounds derived from structural analysis of the target oncoproteins or their co-factors. Recombinant fragments of the target protein are then used to screen the chemical combinatorial libraries for compounds which exhibit high affinity or inhibitory activity for the target proteins. In addition, scaffolds are used which are attached to the lead compounds or are mimics for the natural ligands for the target pockets. These scaffolds, which project chemical functionalities in multiple directions, can be subjected to combinatorial structural diversification (18). Then, when the library is screened with recombinant clones of the target protein for high affinity binding, the ideal compounds are binding the target at multiple independent protein amino acid motifs. This may produce inhibitory compounds which can inhibit target proteins by binding at multiple sites. This theoretically can produce inhibitors which are very selective and high affinity binders.

DESIGN OF MOLECULAR THERAPEUTICS WHICH DO NOT DEPEND ON AVAILABILITY OF PROTEIN STRUCTURE. The algorithm outlined above for the development of drugs based on the analysis of protein structure is different when there is no X-ray crystallographic data or nuclear magnetic resonance imaging based on structural data. The approach to the design of cancer treatment drugs without structural data is based on two approaches.

1. Use of computational chemistry for the design of mimics of co-factors, like ATP for kinase, or substrates which provide information about what kinds of chemical functionalities will

target and bind to the target catalytic sites of the oncoprotein targets. Another example is the development of mimics of low molecular weight ligands for growth factors for growth factor receptors. Once chemical mimics are designed for particular targets, then cell line experiments, followed by animal model experiments are carried out to test the safety and efficacy of the candidate compounds.

2. The other basic approach is to produce recombinant versions of the target protein, and then use these fragments for the screening of phage display random peptide libraries in order to collect peptide motifs which exhibit high affinity binding and selective binding to the target protein. The next step is to identify the common amino acid motifs in the peptides which confer binding specificity to the oncoprotein targets. Next is to use site directed mutagenesis to identify which amino acid moieties are necessary for binding. On the basis of this data, then computational chemistry is used to propose chemical functionalities as mimics of the peptide motifs for the design of low molecular weight chemical functionalities which can bind to and block the function of the oncoprotein upon which the transformed phenotype of the cancer cell depends.

As in the above description of the algorithm used when there is structural data, at the very end, combinatorial libraries can be generated which are based on scaffold molecules which are themselves mimics of the binding domains of peptides which bind to the target proteins. Then, the combinatorial libraries can be generated which contain the bias of the lead compound as the binding domain, but also contain 4 or 5 other chemical functionalities, each projecting from a different position on the scaffold, which can be independently varied structurally. This then creates inhibitors which are binding to 4 or 5 local independent domains at the target site. The chemical inhibitors thereby produced can often bind with very high affinity and very selectively.

Using this approach for drug design, low molecular weight chemicals are being developed for the following targets: growth factor receptors, membrane G proteins, metastasis receptors, signal transduction molecules, transcription factors and transcriptional initiation complexes, replication complexes of DNA tumor viruses, and DNA tumor viruses transforming proteins. In addition, normal protein targets such as anti-angiogenesis inhibitors are being developed as well.

APPROACHES FOR THE DESIGN OF MOLECULAR THERAPEUTICS FOR GYNECOLOGICAL DISEASES

DESIGN OF MOLECULAR THERAPEUTICS FOR HPV ASSOCIATED NEOPLASMS. Human papilloma virus (HPV) associated neoplasms represent the second leading cause of cancer death among women in the world (19). In developing nations, HPV asso-

which are inhibitory for growth factor receptors

high risk genotypes of

ciated neoplasms represent the leading cause of death among women between the ages of 25 and 35. In the United States, cervical cancer has been controlled for the majority of individuals who participate in yearly screening with the PAP smear. ~~Detection of~~ chronic dysplasia associated with ~~chronic~~ HPV infections which place women at a very high risk of developing cervical cancer. However, this transition from a dysplastic mucosa, in which the HPV DNA is replicating as an episome, and frankly invasive cancer, in which the HPV DNA is integrated into the host cell DNA, may require years.

possibly Most people clear the HPV infection spontaneously. However, a small percentage of individuals fail to clear the infection due to a ~~cryptic~~ defect in antigen presentation. These are the individuals in whom there is a high risk of developing cervical cancer. The PAP smear picks up these individuals. If the dysplasia is severe, surgical interventions are utilized to treat these individuals. In developing nations, there is no infrastructure for the screening of individuals who have cervical dysplasia or who are positive for chronic HPV infections.

are Among immunodeficient individuals, ~~the~~ HPV infection ~~is~~ much more extensive and may spread to the rectal and anal mucosa. ~~The~~ Chronic infection in immunodeficient individuals places the individual at risk of developing rectal cancer. A PAP smear for the rectal mucosa has been developed for HPV infections of these individuals as well.

Two approaches are being used *by* to attempt to prevent cervical cancer: vaccines which would make women and men all over the world resistant to infection ~~with the~~ HPV, and drugs which are specifically targeted to suppress the replication of the HPV virus.

replication At Yale, Computational chemistry was used to make predictions about the types of chemicals which might suppress the replication of the HPV through disruption of the ~~transcription~~ initiation complex on the LCR of the viral DNA in the laboratory of David Austin. These data indicated that the indole nucleus might be useful as an inhibitor of replication of HPV, which depends on E2 ~~and E1~~.
which depends

Fujii *et al.* (20), working in the Deisseroth laboratory, ~~also~~ found that the indole nucleus appeared in random peptides which ~~were found to~~ bind to the HPV E2 nucleus. This work suggested that the indole nucleus would be important as a basis on which to build low molecular weight chemical inhibitors of HPV replication. ~~In fact, the data developed in the laboratories of Austin and Deisseroth led to the funding of a multi-institutional National Cancer Institute trial designed to study the feasibility of using the indole nucleus to suppress chronic infections associated with HPV. At the same time, plans were developed in the Austin laboratories to use combinatorial and~~

computational chemistry to develop new inhibitors for the replication of HPV (18).

DEVELOPMENT OF MOLECULAR THERAPEUTICS BASED ON REPLICATION COMPETENT ADENOVIRAL VECTORS FOR OVARIAN CANCER Ovarian cancer is restricted to the peritoneal cavity in the majority of patients even at advanced stages of disease (21). However, 80% of patients are unresectable due to the dissemination of the ovarian cancer from the primary tumor to multiple sites within the peritoneal cavity as microscopic disease in multiple areas throughout the peritoneal cavity. These microscopic implants ~~define the ovarian cancer patient to relapse even though the gross disease is resectable.~~

lead to relapse in

Intracavitary chemotherapy and abdominal radiation therapy have both failed because the ovarian cancer cells are more resistant to the effects of chemotherapy and radiation therapy than are the normal peritoneal lining cells. With the advent of adenoviral vectors and genetic therapy, there is an expectation that ovarian cancer could be treated with genetic or molecular therapy.

represent this from happening in the majority of patients

Our group has developed a series of adenoviral vectors designed to exhibit selective toxicity towards ovarian cancer without damaging the normal peritoneal lining cells of the peritoneal cavity. The start of this project was with the discovery made by Chung *et al.* (22) that the L-plastin promoter, when placed in the backbone of the adenoviral vectors, retained its selectivity of expression on estrogen dependent tumor tissue, and was not actively expressed in the normal peritoneal lining cells (23). This promoter was then placed 5' to the E1A gene of the adenoviral vector, which the vector needs to be replication competent and directly ~~lytic to~~ tumor cells (24). This produced a vector which was directly cytolytic to ovarian cancer cells, but which was not toxic to normal peritoneal lining cells (24). The next stage was to place a prodrug activation transcription unit in the conditionally replication competent L-plastin/E1A adenoviral vector. Finally, the next phase will be to engineer the fibrillar protein so that the adenoviral vector will bind to tumor cells but not to the normal peritoneal cells.

lyse

SUMMARY OF MOLECULAR APPROACHES TO CANCER TREATMENT

It is clear that the advent of genetics, molecular biology and the ability to transfer structural information about oncoproteins into chemical functionalities, has forever changed the prospects for the development of a new generation of therapeutics which will exhibit greater degrees of selectivity and greater efficacy. Already, the vanguard of this new generation of therapeutics has reached the clinic. The early success with these ~~drugs~~ has raised the hopes that these compounds will produce dividends for cancer patients all over the world. In addition, the increased selectivity of these compounds will re-

agents

These vectors are being studied in pre-clinical animal models.

duce the suffering of patients and reduce the burden imposed on society due to toxicity.

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REFERENCES

1. Folkman J. Tumor angiogenesis: therapeutic implications. *N Engl J Med* 1971; 285:1182-1186.
2. Jacobson VS, Stolz DB, Schnitzer JE. Identification of endothelial cell-surface proteins as targets for diagnosis and treatment of disease. *Nature Medicine* 1996; 2:482-485.
3. Callender NS, Varki N, Rao LVM. Immunohistochemical identification of tissue factor in solid tumors. *Cancer* 1992; 70:1194-1201.
4. Nemerson Y. Tissue factor and hemostasis. *Blood* 1988; 71:1-8.
5. Pasqualini R, Ruoslahti E. Organ targeting in vivo using phage display peptide libraries. *Nature* 1996; 380:364-366.
6. Pasqualini R, Koivunen E, Ruoslahti E. Alpha v integrins as receptors for tumor targeting by circulating ligands. *Nature Biotechnol* 1998; 15:542-546.
7. Sauter BV, Martinet O, Zhang WJ, Mandeli J, Woo SLC. Adenovirus-mediated gene transfer of endostatin in vivo results in high level of transgene expression and inhibition of tumor growth and metastases. *Proc Natl Acad Sci USA* 2000; 97:4802-4807.
8. Hu ZW, Sun Y, Garen A. Targeting tumor vasculature endothelial cells and tumor cells for immunotherapy of human melanoma in a mouse xenograft model. *Proc Natl Acad Sci USA* 1999; 96:8161-8166.
9. Hu AW, Garen A. Intratumoral injection of adenoviral vectors encoding tumor-targeted immunoconjugates for cancer immunotherapy. *Proc Natl Acad Sci USA* 2000; 97:9221-9225.
10. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. *Nature* 1998; 392:245-252.
11. Choudhury A, Gajewski JL, Liang JC, Popalt U, Claxton DFR, Kliche KO, et al. Use

of leukemic dendritic cells for the generation of antileukemic cellular cytotoxicity against Philadelphia chromosome-positive chronic myelogenous leukemia. *Blood* 1997; 89:1133-1139.

12. Kolb HJ, Schattenberg A, Goldman JM, et al. Graft versus leukemia effects of donor lymphocyte transfusions in marrow grafted patients. *Blood* 1995f; 86:2041-2048.
13. Bevin MJ. Cross-priming for a secondary cytotoxic response to minor H antigens with H-2 congenic cells which do not cross-react in the cytotoxic assay. *J Exp Med* 1976; 143:1283-1288.
14. Huang TH, Wu PY, Lee CN, Huang HI, Hsieh SL, Jung J, et al. Enhanced antitumor immunity by fusion of CTLA-4 to a self tumor antigen. *Blood* 2000; 96:3663-3670.
15. Lin KY, Guarniere FG, Stveley-O'Carroll KF, Levitsky HI, August JT, Pardoll DM, Wu TC. Treatment of established tumors with a novel vaccine that enhances major histocompatibility class II presentation of tumor antigen. *Cancer Res* 1996; 56:21-26.
16. Gong J, Chen D, Kashiwaba M, Kufe D. Induction of antitumor activity by immunization with fusions of dendritic and carcinoma cells. *Nature Med* 1997; 3:558-561.
17. Thiesing JT, Ohno-Jones S, Kolibaba KS, Druker BJ. Efficacy of STI 571, an Abl tyrosine kinase inhibitor, in conjunction with other antileukemic agents against Bcr-Abl-positive cells. *Blood* 2000; 96:3195-3199.
18. Deisseroth A, Psyrri A, Austin DJ. Molecular Therapy. In: Principles and Practice of Oncology. DeVita VT Jr, Hellman S, Rosenberg SA, eds. 6th edn. Lippincott-Raven, New York, 2000: 3180-3187.
19. Parkin DM, Pisani P, Ferlay J. Estimates of the worldwide incidence of 25 major cancers in 1990. *Int J Cancer* 1990; 80:827-841.
20. Fujii T, Austin D, Deisseroth A. Peptide ligands for HPV 16 E2 isolated from a random peptide library. Submitted 2000.
21. McGuire WP, Hoskins WJ, Brady MF, et al. Cyclophosphamide and cisplatin compared with preclinical and cisplatin in patients with stage III and stage IV ovarian cancer. *N Eng J Med* 1996; 334:1-6.
22. Chung I, Schwartz PE, Crystal RG, Pizzorno G, Leavitt J, Deisseroth AB. Use of L-plastin promoter to develop an adenoviral system that confers transgene expression in ovarian cancer cells but not in normal mesothelial cells. *Cancer Gene Therapy* 1999; 6:99-106.
23. Peng XY, Rutherford T, Wong H, Pizzorno G, Sapi E, Kaczinski B, et al. The use of the L-plastin promoter for adenoviral mediated tumor specific gene expression in ovarian and bladder cancer cell lines. Submitted, 2000.
24. Zhang L, Peng XY, Pizzorno G, Deisseroth A. Tumor specific promoters confer tumor specific cytolytic action on adenoviral vectors in estrogen dependent tumors and melanoma. Submitted, 2000.

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